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Footnotes. These should be avoided in the text as far as possible. Where they must be used, as in tables, reference is made by the symbols *†‡§‖¶, in that order.

A Thermostat Cell-holder for the Beckman Spectrophotometer

By M. DIXON
Department of Biochemistry, University of Cambridge

(*Received* 22 *August* 1953)

The Beckman spectrophotometer is very convenient for following the progress of many enzyme reactions by measuring the change of absorption spectrum which accompanies the reaction. For this purpose it is necessary that the temperature of the cell should be kept accurately constant in the spectrophotometer in spite of the heat from the neighbouring lamp-house. Moreover, for studies on enzyme kinetics it is important to be able to work at different temperatures covering a wide range from 0° upwards.

Several temperature-controlled cell-holders have been devised for this purpose, e.g. that of Bell & Stryker (1947), consisting usually of some form of water-jacket through which water is circulated from an external thermostat. In all of these, however, so far as the writer is aware, the circulating water does not come into contact with the cells themselves, but the cells are placed in a well in the holder in such a way that they are surrounded by a layer of air. This tends to insulate the cells thermally from the surrounding jacket and greatly prolongs the time necessary for the cell contents to reach the desired temperature. Bell & Stryker, for instance, estimate that an equilibration period of 10 min. is necessary. This is a serious drawback for enzyme work.

This paper describes an easily constructed holder in which the cells are actually immersed directly in the circulating water, as a result of which the rate of thermal equilibration has been increased about tenfold. This device has been in constant use for the past two years for work on enzymes by a number of workers in this department, especially for studies on fumarase kinetics by Massey (1953), and it has proved so useful that it has been thought worth while to publish details of its construction.

It consists essentially of a Perspex box surrounding two standard 10 mm. quartz cells (Figs. 1, 2); watertight joints between the cells and the box are made by means of compressed rubber gaskets. The holder is suspended by rigid brackets from a brass plate which forms the lid of the spectrophotometer cell chamber and which can be slid so as to bring either cell in turn into the path of the light beam. The lid carries on its under-surface another brass plate, the ends of which act as stops; these are cut so that when either end is brought up against the wall of the chamber the light beam passes through the centre of the corresponding cell. Two rubber tubes for the circulating water are brought down through closely fitting holes in the lid to the cell-holder. The holder is hinged to the supporting brackets so that when it is removed from the chamber the lid can be swung down to the side to allow free access to the cells for filling. A small stand fitting the bottom of the holder loosely is useful to support it when it is removed from the instrument.

The problem of providing windows in the water jacket which do not absorb in the ultraviolet has been avoided by having nothing in the path of the light beam but the quartz cells themselves and their contents. This has been achieved by providing the window apertures in the front and back of the holder with sliding frames carrying rubber gaskets on their inner ends. These are so arranged that when the frames are pushed home the gaskets press on to the cells to make a watertight joint, and at the same time the pressure causes them to expand sideways and press against the sides of the apertures, so sealing them. The frames have projecting flanges within the gaskets which direct the pressure outward and prevent them from bulging into the light path.

Where the cells pass through the top of the holder, watertight joints are similarly made by compression of rubber gaskets contained in the apertures in plate *B* by screwing down plate *A*; this causes them to press both on to the top of the box and on to the cells, so making a complete seal.

The cells can be withdrawn for cleaning purposes simply by releasing the pressure on the gaskets, though this has not been found necessary in practice.

Since the holder is 3 cm. in thickness, it is necessary to replace the cell chamber of the spectrophotometer by another made up from three pieces of $\frac{1}{8} \times 1\frac{3}{16}$ in. brass bar, fastened together with screws and soldered at the corners, and drilled to fit the locating pins and bolts of the Beckman spectrophotometer. It is convenient to have an inlet at the bottom for a stream of dry air to prevent the cells from steaming up when working at 0°. It will be necessary to replace the four bolts holding the photocell compartment by longer ones.

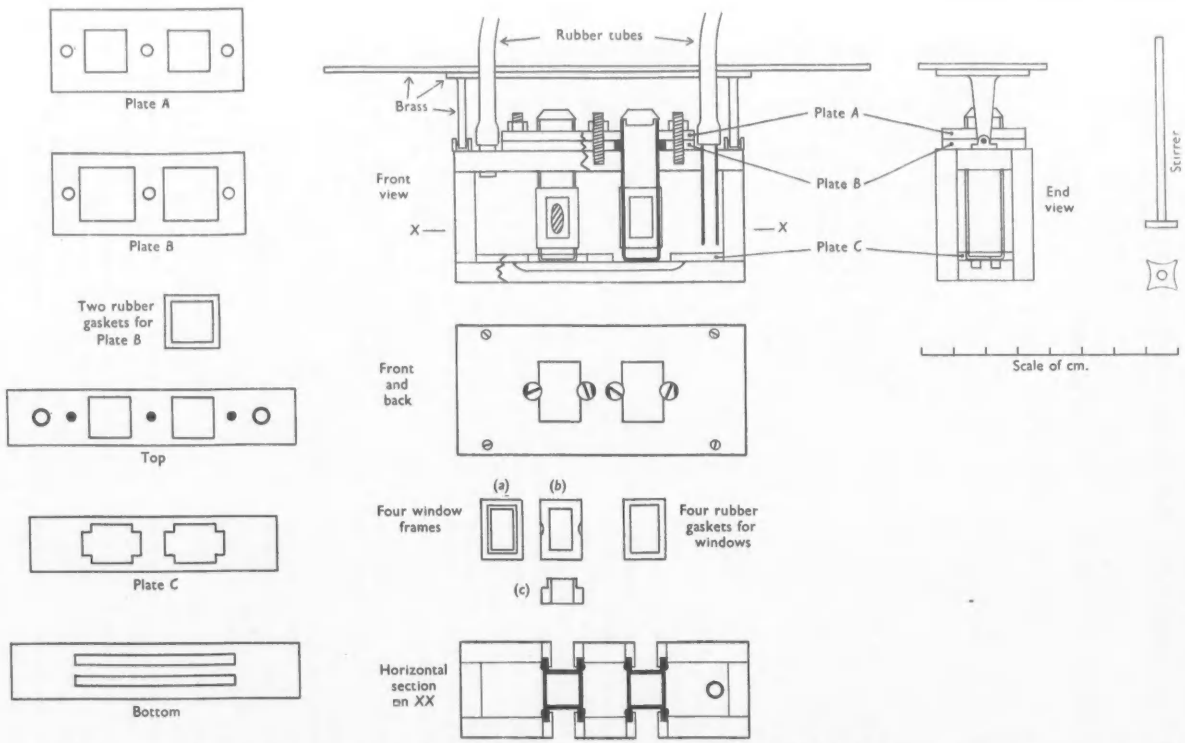

Fig. 1. Working drawings of cell-holder and its parts (half actual size). *Front view* (shown partly in section): the shaded area shows the position of the widest part of the light beam; with the Beckman instrument used this was obtained with a distance of 6 cm. between the lower surface of the top brass lid and the upper surface of the bottom plate of the holder, but this should be checked with the instrument for which the holder is intended; the dimensions of the lid are 17 cm. × 4 cm. × $\frac{1}{16}$ in. The dimensions of the other parts are as follows. *Front and back plates*: 9 cm. × 41 mm. × $\frac{1}{4}$ in., with two rectangular holes 19 mm. × 13 mm. with centres 13 mm. from centre lines and lower sides 10·5 mm. from bottom edge of plate. *Four window frames* ((*a*) back view, (*b*) front view, (*c*) horizontal section) 19 mm. × 13 mm. × $\frac{3}{16}$ in., pierced with holes 13 mm. × 7 mm. and with a 2 mm. × 3 mm. rebate cut round one face. *Four rubber gaskets* 19 mm. × 13 mm. with holes 15 mm. × 9 mm., cut from a $\frac{3}{16}$ in. sheet of soft rubber with smooth surface. *Plate A* 6 cm. × 26 mm. × $\frac{1}{8}$ in., with two holes 13 mm. square and three 4 BA clearance holes. (The centres of the square holes in plates *A–C* and the top are all at 13 mm. from the centre line, and the 4 BA holes in plates *A* and *B* and the top are spaced 25 mm. apart.) *Plate B* 6 cm. × 26 mm. × $\frac{1}{8}$ in., with two holes 17 mm. square and three 4 BA clearance holes. *Two rubber gaskets* 17 mm. square, with holes 13 mm. square, cut from $\frac{3}{16}$ in. sheet. *Top Plate* 9 cm. × 17·3 mm. × $\frac{1}{4}$ in., with two holes 13 mm. square and two holes to fit the Perspex tubes, and with three 4 BA brass studs tapped in. *Plate C* 77·3 mm. × 17·3 mm. × $\frac{3}{32}$ in., with two holes 13 mm. square with extensions as shown. *Bottom plate* 9 cm. × 17·3 mm. × $\frac{1}{4}$ in., with two grooves about $\frac{1}{8}$ in. × $\frac{1}{8}$ in. milled in upper surface. *Two end plates* (not shown) 28·3 mm. × 17·3 mm. × $\frac{1}{4}$ in.

CONSTRUCTIONAL DETAILS

The parts of the Perspex box are preferably squared-up accurately to size in the milling machine. Two slots are milled in the upper surface of the bottom piece as shown to permit some of the water to pass below the cells. The window frames should be an easy sliding fit in the apertures and are held in position by small brass screws with flat heads 6 mm. in diameter. The tapped holes for these must not pass right through the front and back pieces. The heads are recessed into these pieces and into the window frames in such a way that when the screws are home the heads are flush with the outside of the holder and the frames are also held flush with the front and back surfaces. After fitting, the frames are removed, the screws replaced and driven home, and then unscrewed for half a turn. The part of the heads which overlaps the window apertures is then removed with a file. This enables the window frames to be released later for removing the cells by a half turn of the screws.

The top is drilled and tapped for three 4 BA brass studs as shown, care being taken that the holes do not penetrate through to the under side. It is also drilled to fit the two pieces of Perspex tube accurately and these are fixed in position with Perspex cement. Plate *C*, which locates the bottoms of the cells, is stuck on to the bottom piece with Perspex cement.

The holder is then assembled by coating the joining surfaces with Perspex cement and immediately fastening firmly together with screws, so as to obtain watertight joints throughout.

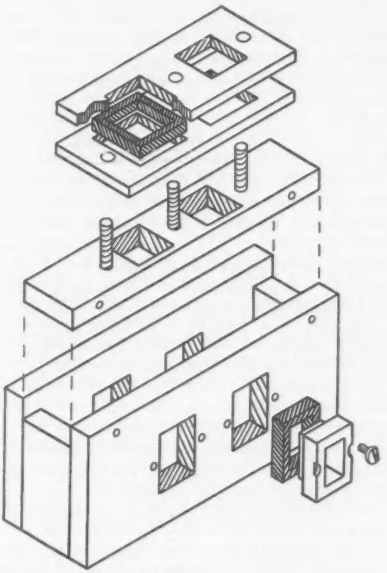

Fig. 2. Isometric drawing of cell-holder showing details of assembly.

Plate *B* with its two rubber gaskets is placed in position on the brass studs, followed by Plate *A*. 4 BA nuts are placed on the studs, but not tightened. The quartz cells are then placed in position and the nuts tightened so that they are held firmly. The gaskets should be cut so that when the nuts are screwed down the cells are quite firmly held, but without excessive pressure on their faces. If necessary, the pressure may be adjusted by placing thin washers on the studs between Plates *A* and *B*.

Finally the window frames, with their rubber gaskets, are pressed in from both sides simultaneously and secured by their screws. The whole jacket should then be watertight.

The water jacket is attached by hinges to the suspending brackets fixed to the lid. The photocell assembly is then removed from the spectrophotometer and the light turned on. The cell compartment is held in position and the cell-holder placed in it. It is then easy to verify that the ends of the brass plate on the under side of the lid have been cut correctly so that the light beam is accurately centred in the window frames when the lid is slid as far as it will go in either direction.

Some difficulty was met with in cutting the rubber gaskets until the following method was devised. A wafer-type safety razor blade is broken off to a length about $\frac{1}{2}$ mm. longer than the cut desired. The breaks are made at an angle of about 80° so that the cutting edge is the widest part. The piece of blade is held firmly in a small hand vice. The sheet of soft rubber is placed on a flat wooden surface, the edge of the blade is carefully placed in the desired position and the blade is pressed straight down through the sheet without any sawing motion. As the blade is a little too long, the cuts cross slightly at the ends and give square corners. None of the pieces of rubber should be removed from the sheet until all the cuts have been made, otherwise there will be some distortion of the rubber during cutting. For the same reason a margin should be left unused at the edge of the sheet.

Water is circulated from an external thermostat of about 3 l. capacity by means of a no. 10 Stuart centrifugal pump run with a speed-controlling resistance in series. A rate of about 300 ml./min. is quite adequate.

A small Perspex stirrer as shown is useful for quickly mixing the cell contents. It is shaped so that it cannot come into contact with the optical faces of the cells.

SUMMARY

A description is given of a cell-holder for the Beckman spectrophotometer in which the temperature of the cells is held constant by water circulating in contact with them. This gives rapid equilibration and accurate control.

The cell-holder was made for me by Mr H. Overhill in this department. I am indebted to him for the care which he took in its construction.

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The Action of Proteolytic Enzymes on *Clostridium botulinum* type A Toxin

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Clostridium botulinum toxin is one of the most toxic substances known, the type A toxin containing about $220–250 \times 10^6$ mouse LD_{50}/mg. N (Putnam, Lamanna & Sharp, 1948). It is a protein of molecular weight about one million whose amino acid composition (Buehler, Schantz & Lamanna, 1947) as yet offers no indication of its mode of action.

As early as 1923, Schubel noted that the toxicity of culture filtrates of the organism was more sensitive to alkali than to acid and also was unaffected by pepsin and trypsin. Such observations have been confirmed repeatedly by other workers (Bronfenbrenner & Schlesinger, 1924; Nelson, 1927; Snipe & Sommer, 1928; Chistyakov & Rodopulo, 1943; Littauer, 1951), whilst Kalmanson & Bronfenbrenner (1943) reported that papain caused no destruction of the toxin.

These observations were concerned with crude toxin preparations but even with the crystalline material Abrams, Kegeles & Hottle (1946), Putnam *et al.* (1948) and Lamanna, Eklund & McElroy (1946) found that the protein is rapidly destroyed above pH 7, whilst Putnam *et al.* (1948) also recorded that 'even mild physical treatment of pure toxin produced loss of activity, particularly at high dilutions'.

In the present investigation the stability of partially pure and crystalline toxins has been examined at various pH values. The action of the crystalline enzymes, pepsin, trypsin and papain on the pure toxin has also been studied.

EXPERIMENTAL

Materials

Cl. botulinum *toxin*. Sarles & Herbert (1943, unpublished) reported that when cultures were allowed to stand at room temperature, after the usual period of incubation, much of the toxin was adsorbed on the fine precipitate of cell debris which settled out. The toxin can be recovered from the centrifuged deposit by extraction with an acid citrate buffer containing 0·18M sodium dihydrogen citrate, 0·02M disodium hydrogen citrate and M-NaCl, giving a pH of 4·0 (Bottomley & Creeth, private communication). The details whereby the toxin can be finally brought to the crystalline stage involve $(NH_4)_2SO_4$ fractionations after Lamanna, Eklund & McElroy (1946). All toxin specimens were prepared by extraction of the lysed culture precipitate, but whereas the first samples were crystalline their activities (about 70×10^6 LD_{50}/mg. N) were only about 30% of that quoted by Putnam *et al.* (1948), though later batches provided toxin with activity (250×10^6 LD_{50}/mg. N) comparable to that of the material isolated in the American laboratories (Putnam *et al.* 1948).

The two forms of crystalline and electrophoretically homogeneous toxin described above are hereafter termed 'partially pure' (low activity) and 'pure' toxin (high activity). Samples of both forms were used for investigating the stability of the toxin but only 'pure' toxin samples were employed in the enzymic work.

Enzyme preparations. Crystalline pepsin and trypsin were purchased (Armour Biochemicals) and their activities were determined according to Northrop, Kunitz & Herriot (1948) with haemoglobin. Crystalline papain was prepared and assayed according to Balls & Lineweaver (1939).

Methods

Non-protein nitrogen (N.P.N.). Sodium trichloroacetate, prepared by addition of alkali to the free acid to pH 2·0, was added to a sample of enzymic digest to give a final concentration of 0·25N trichloroacetate pH 2·0, under which conditions precipitation of toxin occurred rapidly. Free trichloroacetic acid could not be used because it inactivated the toxin. The sample was then centrifuged for 10 min. and subsequently filtered, or alternatively it was allowed to stand 0·5 hr. at 0° (low temperature to preserve toxicity) before filtration (Whatman no. 3 paper). As both procedures gave similar values for N.P.N. in the filtrate, the second method was finally adopted in view of its convenience. The filtrate was then adjusted to pH 6·0 and stored at 0° until assayed for toxicity.

Total nitrogen. Digestion for 4 hr. with 1 ml. 18N-H_2SO_4 containing 2·5 mg. $CuSO_4$, $5H_2O$, 20 mg. K_2SO_4 and 2 mg. Na_2SeO_4 was necessary in order to obtain 100% recoveries with not more than 70 μg. N. All reagents were of micro-analytical reagent standard except the Na_2SeO_4 (N-free grade). Distillations were performed in the Markham (1942) apparatus and titrated with barium hydroxide from a Conway microburette.

Ninhydrin values. Owing to the small quantities of toxin available the spectrophotometric method of Moore & Stein (1948) was employed, taking the total unfractionated digest at pH 5·0, with a heating time of 30 min.

Toxicity determinations. Samples for assay were diluted in gelatin–phosphate buffer (Lamanna & Glassman, 1947) and 0·5 ml. was injected intraperitoneally into male albino mice of body weight about 20 g., using an average of nine animals for each dilution. The responses were examined by probit analysis and potencies expressed as LD_{50}/ml.

pH *determinations.* All readings were made with the glass electrode and rechecked at the end of the experiments. Unless otherwise stated citrate–phosphate buffer (McIlvaine) was used to obtain the required pH values.

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General procedure. The section of the work employing 'pure' toxin and crystalline enzymes involved the use of about 100 μg. toxin N/ml. and of 2–4 μg. enzyme N/ml. in citrate–phosphate buffer unless otherwise stated. At intervals, samples were removed into trichloroacetate (for N.P.N. estimation) and also into cold buffer solution, at a suitable pH to inhibit enzymic activity, e.g. for pepsin pH 6·0 and for trypsin pH 5·0. Hoover & Kokes (1947) have shown that papain is active over a wide range of pH values and hence, in the present investigation the papain–toxin solutions were adjusted to an arbitrary pH value of 5·0. All samples were diluted with cold buffer and immediately stored at 0° until assayed for toxicity. These solutions were analysed also for total N to confirm the accuracy of recovery at these stages.

RESULTS

The stability of the toxin in citrate–phosphate buffers at various pH *values*

Samples of the two forms of crystalline and electrophoretically homogeneous toxin described above, i.e. 'partially pure' (low activity) and 'pure' toxin (high activity), not only exhibited differences in activity but also in stability under various pH conditions.

A sample of 'partially pure' toxin solution (17×10^6 LD_{50}/ml.) in 3·5N-HCl at 16·2° retained only 1% of its original activity after 2 min. In 5 hr. the remaining toxicity had also disappeared but no more N.P.N. was released than from the original untreated toxin solution after precipitation directly with trichloroacetate. Solutions of both forms of toxin (2×10^6 LD_{50}/ml.) appeared to be stable at 0° in citrate–phosphate buffer; the 'partially pure' material at pH 6·5 exhibited no loss of toxicity in 8 days whilst a similar retention of activity was shown by the 'pure' toxin at pH 5·0 for at least 40 days. On the other hand, both forms of toxin at this concentration lost all activity after 20 min. at 99°.

The stability of the 'partially pure' material was examined at the same concentration (2×10^6 LD_{50}/ml.) in citrate buffer pH 7·0 and 30° as a preliminary to the use of trypsin; the results in Fig. 1 show rapid loss of activity.

'Pure toxin' (2×10^6 LD_{50}/ml.) was similarly examined for stability. At pH 7·0 and 25° and pH 7·0 and 30·0° no loss was detected up to 26 hr., or at pH 7·0 and 38° in 2 hr. Further information on the greatly increased stability of the 'pure' toxin is given by the control experiments conducted with trypsin at pH's 6·6 and 7·6 (see later).

Action of proteolytic enzymes on the 'pure' toxin

Pepsin. The toxin, adjusted to pH 2·0 with dilute HCl and containing crystalline pepsin (2% of toxin on N basis), was incubated at 29·6° for the periods shown in Fig. 2.

A probit analysis of the unfractionated solutions indicated that the LD_{50} was very similar for all control (toxin and buffer only) solutions and for the toxin solution containing enzyme tested immediately after mixing. Samples of toxin incubated with the enzyme showed a significant fall in toxicity within 3 hr., the level then remaining virtually constant up to 21 hr. Statistical analysis of the results of the toxicity determinations indicated that the difference between the mean values of the untreated and combined pepsin-treated (3–21 hr.) toxins were significantly different at the 1% level and demonstrated a loss of about 25% toxicity in presence of pepsin. The N.P.N. fractions of both control and experimental solutions had no toxicity.

The N.P.N. in the control remained steady at a small value during the incubation. On the other hand, in the peptic digest the N.P.N. increased rapidly and followed closely the loss of toxicity in this period. Therefore the initial value for N.P.N. in the peptic digest of Fig. 2 was calculated from the control solution. The identical values obtained for total N in the first samples for control and experimental solutions show that little error could have resulted from this assumption.

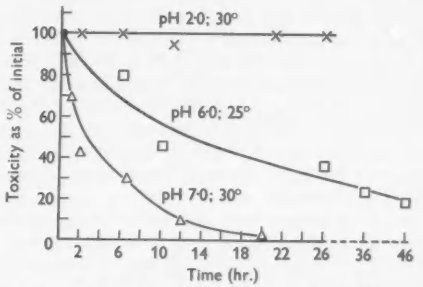

Fig. 1. Effect of pH and temperature on 'partially pure' toxin.

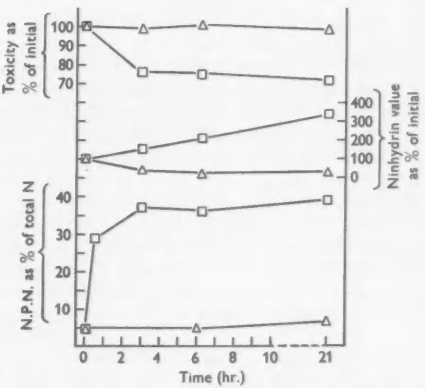

Fig. 2. Effect of pepsin on 'pure' toxin at pH 2·0 and 30°. △, toxin only; □, toxin and enzyme.

Trichloroacetate fractionation provides only limited information on the possible action of enzymes on proteins as degradation to molecules still large enough to be insoluble in trichloroacetate may have occurred. The course of the reaction was therefore also followed with the ninhydrin method because of its suitability for the small amounts of toxin concerned. Fig. 2 indicates an increase in ninhydrin value in the early stages of digestion at the same time as the N.P.N. is increasing. However, the extent of the rise in ninhydrin value suggests that the products formed in the N.P.N. fraction are not small peptides (cf. Moore & Stein, 1948; Dowmont & Fruton, 1952). Even when the N.P.N. reaches its maximum and constant level the ninhydrin value is still increasing.

No explanation is available for the decrease in ninhydrin-positive material in the control which had been kept at pH 2·0 and then brought to pH 6. However, it is known that changes in the dissociation of *Cl. botulinum* toxin occur in solution between pH 2·0 and 4·3 (Wagman & Bateman, 1951).

Trypsin. Previous reports had indicated that crystalline toxin was more stable on the acid side of pH 7·0; therefore in the present work with 'pure' toxin the action of trypsin (4 % of toxin on N basis) was first investigated in citrate–phosphate at pH 6·6 and 25·5°. Although this pH is below the accepted pH optimum for the enzyme the activity was found to be about 50 % of that at pH 7·6 when determined according to Northrop *et al.* (1948) with haemoglobin as substrate.

Fig. 3 illustrates that the 'pure' toxin itself is stable under these conditions, no significant loss of activity occurring in 26 hr., whereas in the presence of trypsin rapid loss of activity ensues in 3 hr. followed by a slower but progressive loss of up to about 90 % activity in 26 hr. The N.P.N. fractions, both control and experimental, exhibited no toxic activity at any stage.

The N.P.N. in the control samples like those in the peptic incubation had a low and constant level during the incubation. In the presence of trypsin, however, a slow and progressive increase in N.P.N. began at the end of the rapid phase of inactivation of the toxin.

In the absence of trypsin the ninhydrin determinations indicated a slight continual increase whilst in the experimental digest little or no alteration was seen during the rapid detoxication phase. Subsequently, a sharp rise in ninhydrin value ensued coincident with both a slight inactivation of toxin and with rise in N.P.N. and was followed by a steady decline to slightly below that of the control (see Discussion).

After it had been shown that the 'pure' toxin was stable at about pH 7·0 and higher, the action of trypsin at pH 7·6 and 25·5° was examined. Fig. 4 shows that no loss of activity was apparent at this pH unless trypsin was present in which case about 90 % loss of toxicity occurred as at pH 6·6.

The ninhydrin results in this experiment also closely follow those at pH 6·6. Very little change was found in absence of trypsin and even with enzyme present no great alteration in ninhydrin value was apparent when toxicity was decreasing rapidly. Later, however, a marked increase in ninhydrin-positive material was found simultaneously with a phase of less rapid but significant

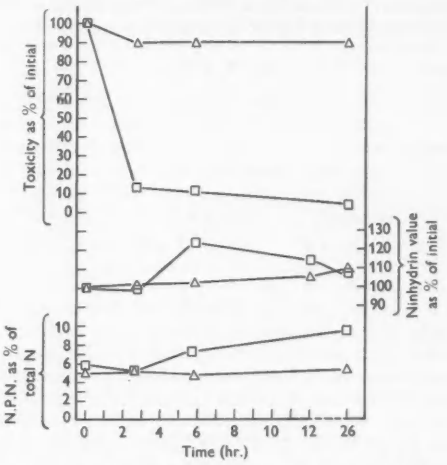

Fig. 3. Effect of trypsin on 'pure' toxin at pH 6·6 and 25°. △, toxin only; □, toxin and enzyme.

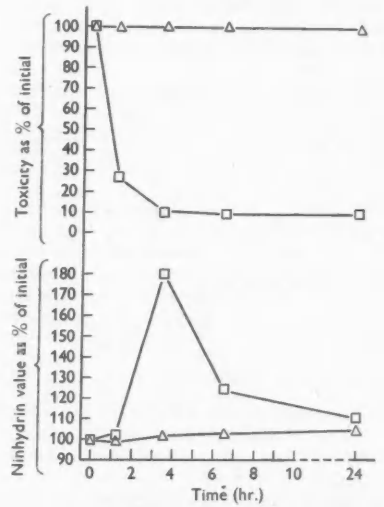

Fig. 4. Effect of trypsin on 'pure' toxin at pH 7·6 and 25°. △, toxin only; □, toxin and enzyme.

detoxication. After this point the toxicity decreased only slightly (cf. Fig. 3) whilst the ninhydrin values gradually diminished (cf. Fig. 3).

Papain. The crystalline enzyme was activated with half its weight of cysteine for 1 hr. at pH 7·0 before mixing (4 % on basis of total N present) with toxin in citrate–phosphate buffer of pH 4·9 at 25·3°.

Fig. 5 indicates that no loss of activity occurred at this pH either in the presence or absence of papain and no toxic activity appeared in the N.P.N. fraction. Likewise, the N.P.N. itself remained constant and of approximately the same value in control and experimental solutions during the whole incubation.

In the absence of papain, the ninhydrin value altered little throughout the experiment, but in the presence of the enzyme an increase was observed in the early stages (in triplicate observations) followed by a fall to a relatively constant value.

DISCUSSION

The rapid inactivation of the 'partially pure' toxin at about pH 7 and its stability at 0° and in acid solutions (pH 2·0) is in agreement with work reported from other laboratories using crude or crystalline preparations of toxin.

The stability of the 'pure' toxin at about pH 7 is contrary to work reported elsewhere either with crude (Littauer, 1951) or crystalline (Abrams *et al.* 1946; Putnam *et al.* 1948) toxins. The toxin employed by the latter groups of workers corresponded somewhat in stability to that of the 'partially pure' toxin used here, which was rapidly inactivated at pH 7.

Examination of the effect of the three enzymes on the toxin shows that papain has no inactivating action, pepsin produces a slight detoxication, whereas trypsin effects almost complete inactivation. The result with papain is similar to that reported elsewhere with crude preparations of toxin (Kalmanson & Bronfenbrenner, 1943), whereas the slight initial loss of activity that occurs with pepsin seen here was not apparent with the impure toxins used by earlier investigators. Likewise, previous reports on the lack of action of trypsin on unpurified preparations of toxin could not be confirmed when crystalline enzyme and 'pure' toxin were employed. Such differences may perhaps be due to the presence of protective impurities in some impure samples of toxin.

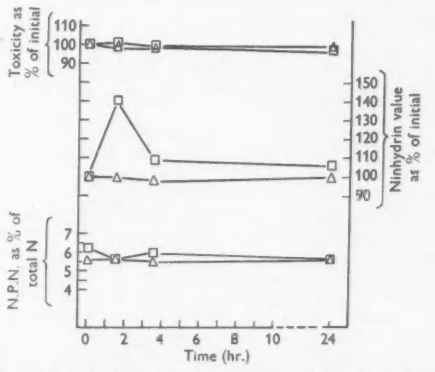

Fig. 5. Effect of papain on 'pure' toxin at pH 4·9 and 25°. △, toxin only; □, toxin and enzyme.

In none of the enzymic experiments with papain, pepsin and trypsin could toxicity be detected in the soluble N.P.N. fraction. Only in the case of the peptic digest did the N.P.N. rise much higher than in the control without enzyme. In view of the evidence of Lamanna & Lowenthal (1951) that type A toxin consists of two components—a haemagglutin and a true toxin—it is possible that in the present work pepsin is primarily attacking the haemagglutin rather than the toxic factor. By this means a large increase in N.P.N. would be achieved with a possible slight loss in toxicity.

The ninhydrin values in the experiments with trypsin and papain are of particular interest in that with the former enzyme the ninhydrin value, like the N.P.N., remains relatively unchanged whilst the most rapid detoxication is occurring. With papain a slight increase in ninhydrin value takes place although no toxic activity is lost. Such observations suggest that loss of toxicity can ensue from minor modification of the original protein and not necessarily by gross degradation of the molecule.

Note added in Proof. Since this work was completed I. W. Coleman (*Canad. J. Biochem. Physiol.* (1954) **32**, 27) has reported the action of pepsin and trypsin on the toxicity of crystalline toxin. In both cases, however, the inactivation was very much smaller than in our experiments, whilst the toxin itself corresponded in purity and stability to our 'impure' material.

SUMMARY

1. Two forms of crystalline *Clostridium botulinum* type A toxin have been prepared. One of these was low in specific activity ('partially pure') and was highly unstable at pH 7·0, whilst the second form possessed much higher specific activity ('pure' toxin) and was more stable at pH 7·0.

2. The action of crystalline proteolytic enzymes on the 'pure' toxin has been investigated. With the conditions adopted, papain had no inactivating effect, pepsin produced slight detoxication whilst trypsin greatly lowered the toxicity. In none of the enzymic experiments was any toxic product of low molecular weight produced.

The author wishes to express his appreciation to Dr D. W. Henderson, Microbiological Research Department, Ministry of Supply, Porton, for facilities to prepare the toxin; to Dr J. Keppie for help in this direction; and to Sir Charles Dodds, F.R.S., and Professor F. Dickens, F.R.S., for interest and support.

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The Antithrombin Activity of Human Plasma

By J. W. LYTTLETON*

(*Received* 15 *February* 1954)

The presence of an agent in serum capable of progressively inactivating the thrombin formed during the coagulation of blood or plasma has long been recognized. After it had been suggested that the factor involved in this action was protein in nature (Rettger, 1909), three main ideas were put forward as to the method of inactivation. Landsberg (1913) considered that thrombin was inactivated by a reversible non-specific adsorption on to serum albumin; Collingwood & MacMahon (1912) held that thrombin was destroyed by a specific enzyme in serum; and Howell (1916) and his co-workers maintained that thrombin was rendered inactive by combination with a specific protein, the product of this combination being termed metathrombin.

More recent work has shown that antithrombin occurs in the albumin fraction of serum as obtained by salt precipitation (Lenggenhager, 1935; Quick, 1938; Stewart & Rourke, 1940; Astrup & Darling, 1942), but the factor itself has not been isolated. Astrup & Darling (1942), working with bovine materials, described a method for the quantitative estimation of antithrombin based on the fact that a given quantity is capable of inactivating only a definite number of thrombin units, and that this inactivation is complete in 15 min. Several observations recorded in the literature indicated that this method of measurement might not be directly applicable to the estimation of human antithrombin. Both Collingwood & MacMahon (1913) and Quick (1938), working with human materials, observed that thrombin inactivation continues for at least 30 min., while Herbert (1940) found that in a given time human serum would inactivate a definite fraction of the thrombin present, independent of its absolute concentration.

These apparent differences between the behaviour of human and bovine antithrombin indicated that a more complete examination of the reaction between human thrombin and human antithrombin might yield new information.

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MATERIALS AND METHODS

Veronal buffer. Unless the contrary is stated, all the dilutions of materials used were carried out with a veronal buffer (Michaelis, 1930) of pH 7·3, $I = 0{\cdot}15$. Two litres of this buffer contained 215 ml. 0·2N-HCl, 14·43 g. NaCl, and 11·74 g. Na barbiturate.

Fibrinogen. The estimation of antithrombin depends on measuring the inactivation of thrombin, and the strength of thrombin is measurable only in terms of the rate of coagulation of fibrinogen, whether this be native fibrinogen in plasma or a solution of fibrinogen purified by fractionation. In these experiments a solution of bovine fibrinogen was used as coagulation substrate, the fibrinogen being obtained by precipitation from bovine plasma by addition of 0·11 vol. ether at 0°. The precipitate was washed with citrate saline containing 8% (v/v) ether, redissolved in citrate saline, and freeze-dried. The fibrinogen obtained in this way appears to be electrophoretically and ultracentrifugally homogeneous. It gives a satisfactory solution for measuring thrombin

activity, and was used in preference to human fibrinogen because it was more readily obtainable in large quantities, and also because, unlike the human material, it became cloudy in the presence of thrombin immediately before coagulation, making accurate timing of the clotting very easy.

Thrombin. Human thrombin was prepared by a method developed from that devised by Kekwick, Mackay & Record (1946) (see also Kekwick & Mackay, 1954). After precipitating the fibrinogen from citrated human plasma by the addition of 0·11 vol. ether at 0° the supernatant solution was adjusted to pH 5·3 by the addition of 0·5 M acetic acid (130–135 ml./l.). The precipitate of crude prothrombin formed at 0° was centrifuged down and washed twice by resuspending in distilled water at 0° and again centrifuging down. The washed precipitate was dissolved in veronal buffer pH 7·3, $I = 0·15$, and reprecipitated by reducing the ionic strength to $I = 0·033$ and then adjusting the pH to 5·3 with 0·1 M acetic acid. After redissolving in citrate saline to 1/10 original plasma volume and neutralizing, this prothrombin was converted into thrombin by addition of human placental thromboplastin and $CaCl_2$. When the conversion was complete, the thrombin was partially purified by adding 0·1 vol. ether at 0° and adjusting the pH to 5·3, when a large proportion of the contaminating impurities was precipitated. The thrombin solution was adjusted to pH 7·3 by dialysis against veronal buffer pH 7·3, $I = 0·15$, and freeze-dried in 10 ml. samples. This provided a suitable standard, effectively free from contamination with antithrombin. The thrombin activity was 93 units/mg. protein N.

Thrombin unit. The unit used was that defined by Owren (1947). According to this definition one thrombin unit is equal to the amount of thrombin which, in a volume of 1 ml. containing 0·10–0·15 g./100 ml. human fibrinogen at 37°, pH 7·3, and $I = 0·15$, due to NaCl, will cause a clot to form in 15 sec. This is a modification of the Iowa unit (Warner, Brinkhous & Smith, 1936) in which the temperature used was 28°.

The veronal buffer used as a diluent in these experiments has been shown by Owren (1947) not to alter the coagulation time of fibrinogen from that in 0·15 M-NaCl, in terms of which solution the thrombin unit is defined.

Measurement of thrombin activity. When solutions of ox fibrinogen are coagulated by human thrombin under standard conditions, the plot of the coagulation time against the reciprocal of the thrombin concentration gives an almost linear relation which can readily be used in measuring the thrombin activity of unknown solutions.

The coagulation was measured in $2 \times \frac{3}{8}$ in. thin-walled test tubes placed in a rack in a glass-walled water bath at 37°. Into each tube was pipetted 0·8 ml. bovine fibrinogen solution, diluted with veronal buffer to contain 0·125 g. protein/100 ml.; after 3 min. temperature equilibration 0·2 ml. thrombin solution, also at 37°, was injected from a capillary pipette by means of an attached hypodermic syringe, a stop-watch being started at the same instant. The tube was spun in its rack to produce a rotatory movement of its contents, which were under continuous observation, until coagulation took place. This was shown by the sudden immobilization of the small bubbles produced in the tube as a result of the rapid injection of the thrombin. With coagulation times of the order of 15 sec., the mean deviation of an individual measurement from the average was 0·2 sec.; this increased to 2·0 sec. with coagulation times from 60 to 80 sec.

Antithrombin. The standard source of active antithrombin was defibrinated human plasma (DFP). A 200 ml. sample of fresh human plasma was taken from a plasma pool from sixty donors, the blood of each donor having been mixed with 120 ml. 3% (w/v) Na citrate solution to give 540 ml. citrated blood. The plasma sample was diluted with an equal volume of veronal buffer, and the fibrinogen removed as fibrin by adding 1 unit of thrombin for every 20 ml. diluted plasma, while stirring rapidly to prevent clot formation. The defibrinated plasma, referred to subsequently as DFP, was freshly prepared every 2 weeks; and since material which had been stored for periods of up to 2 months showed no marked change in antithrombin activity, the antithrombin content of the DFP was taken as constant, and arbitrarily defined as containing 50 antithrombin units/ml.

Examination of antithrombin activity. Antithrombin activity was studied in all cases by incubating a sample of the material with thrombin in veronal buffer at 37° and following the decay of thrombin activity by coagulating a solution of fibrinogen with samples removed at regular intervals. In this way a curve could be constructed of thrombin activity against time, enabling the course of the reaction between thrombin and antithrombin to be readily studied.

Electrophoresis. Electrophoretic analyses were carried out in a Tiselius apparatus in phosphate buffer, pH 8, $I = 0·2$, with protein concentrations of 2% (w/v), determined refractometrically. Optical observations were made by the diagonal Schlieren method (Philpot, 1938), the light source being a high-pressure mercury arc from which monochromatic light, $\lambda = 546$ mμ., was isolated.

The same Tiselius apparatus was used in early work on separating the components of plasma by lateral displacement of the two intermediate sections of the U-tube at the conclusion of a run. In later work the flow-through apparatus (Kekwick, Lyttleton, Brewer & Dreblow, 1951) was used to separate larger quantities of electrophoretically pure components.

RESULTS

Reaction between human thrombin and human antithrombin

Curves showing the thrombin inactivation due to defibrinated plasma (DFP) are given for two typical groups of incubation mixtures. In the first (Fig. 1) the concentration of thrombin was constant and the amount of DFP added was varied; while in the second (Fig. 2) the concentration of thrombin initially present was varied and the amount of DFP held constant.

When the proportion of DFP added to thrombin solutions exceeded a certain value, an immediate prolongation of the coagulation time occurred if the resulting mixture was used to coagulate a solution of fibrinogen. Unlike the progressive antithrombin action with which this paper is chiefly concerned, this immediate prolongation effect was still obtained after heating the DFP to 67° for 10 min. The complication of correcting for this effect, which did not become appreciable until the proportion of DFP in the incubation mixture exceeded 30% (v/v), was avoided by keeping the proportion of DFP

below 20 % (v/v) in the majority of the experiments described. In such a case as curve 6 of Fig. 1, however, it was necessary to obtain calibration curves with fibrinogen solutions containing an equivalent amount of heated DFP, in order to calculate the thrombin concentration from the measured clotting time.

The curves of Figs. 1 and 2 show that under the conditions of the experiment the thrombin concentration seems to approach zero asymptotically at a rate which varies both with the concentration of thrombin and of DFP present. When the amount of DFP added is small, thrombin is still inactivated at a perceptible rate after 200–300 min. (Fig. 1). The type of reaction involved thus differs from that found by Astrup & Darling (1942) for ox blood, and

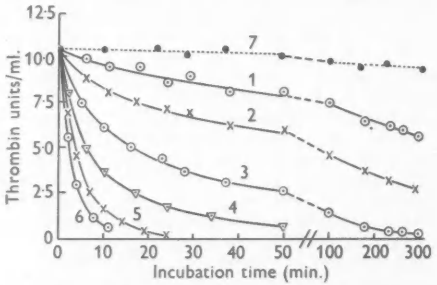

Fig. 1. Action of DFP on thrombin: initial thrombin concentration constant, DFP concentration varied. Mixtures 1–6 contained 1·0 ml. thrombin (53 units/ml.) and the following amounts of DFP: curve (1) 0·1 ml.; (2) 0·2 ml.; (3) 0·4 ml.; (4) 0·7 ml.; (5) 1·3 ml.; (6) 2·0 ml. Control mixture 7 contained no DFP. Volumes adjusted to 5 ml. with veronal buffer.

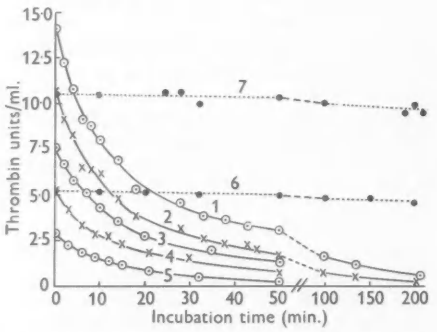

Fig. 2. Action of DFP on thrombin: DFP constant, initial thrombin concentration varied. Mixtures 1–5 contained 0·5 ml. DFP and the following amounts of thrombin (53 units/ml.): curve (1) 0·25 ml.; (2) 0·5 ml.; (3) 0·75 ml.; (4) 1·0 ml.; (5) 1·25 ml. Control mixtures 6 and 7 contained no DFP and 0·5 and 1·0 ml. thrombin respectively. Volumes adjusted to 5 ml. with veronal buffer.

their method of measuring antithrombin in terms of the number of thrombin units inactivated at the end of a reaction reaching completion in a short time (15 min.) is not applicable.

To estimate human antithrombin it is evident that the rate of thrombin inactivation rather than the ultimate amount of thrombin inactivated should be used, for Fig. 1 shows that this rate varies with the DFP concentration. To allow the amount of antithrombin in an unknown mixture to be determined from the decay curve produced when it was added to a standard thrombin solution, it was necessary to discover some property of the curves which could be related quantitatively to the known amount of DFP, or antithrombin, added to the system.

Analysis of decay curves

Kinetics of reaction. The reaction could not be described satisfactorily in terms either of first- or second-order equations. First-order constants (k_1) were calculated for the curves of Figs. 1 and 2, assuming that during any one experiment the antithrombin concentration remained unchanged, and arbitrarily defining an antithrombin unit such that 1 ml. DFP contained 50 units of antithrombin, a definition used in all subsequent calculations.

The values obtained for k_1 decreased from 0·006–0·008 at 5 min. to 0·002–0·003 at 50 min., but the uniformity of behaviour and small random variations in k_1 for the fivefold variation in initial thrombin concentration and 20-fold variation in antithrombin concentration, indicated that the type of reaction occurring was constant within the concentration range considered.

Although an approximately constant value for the second-order reaction constant could be derived from the curves of Fig. 1 by assuming suitable values of initial antithrombin concentration, these values were not proportional to the known amounts of DFP added in each case.

A method for estimation of antithrombin. If analysis in terms of reaction kinetics proves inapplicable to inactivation/time curves, the velocity of the reaction may be measured in terms either of the time required to produce a given percentage inactivation or of the percentage inactivation produced in a given time (Bodansky, 1937). Both of these methods gave values for the reaction velocity which were linearly related to the amount of antithrombin added to the system, provided that the reaction was not nearing completion.

The standard method adopted was to determine from the inactivation curve the percentage of thrombin inactivated in the first 10 min. of the reaction, and to refer this to a calibration curve in which the 10 min. percentage inactivation was plotted against the amount of DFP present in the

system (Fig. 3). This calibration was valid for initial thrombin concentrations of 2–12 units/ml., and enabled the amount of antithrombin present in an unknown system to be determined in terms of DFP. The unit of antithrombin was arbitrarily defined as 1 unit ≡ 0·02 ml. DFP, so in a reaction mixture of 5 ml., 0·1 ml. DFP gives 1 unit antithrombin/ml.

Reduction of antithrombin activity due to incubation with thrombin

The type of reaction between thrombin and antithrombin with its progressively decreasing first-order constant (k_1) suggests that antithrombin is removed from the system as the reaction proceeds. This was confirmed by incubating samples of thrombin solution and DFP together until negligible thrombin activity remained, and then measuring the remaining antithrombin activity (Fig. 4). Initially the amount of antithrombin activity removed increases linearly with the amount of thrombin inactivated, but the rate of removal decreases as the amount removed approaches the total amount present.

As a corollary to this observation it is to be expected that if a large excess of thrombin is added to a given amount of DFP, the antithrombin activity will be reduced to a very low value after a certain amount of thrombin has been inactivated, when the rate of thrombin decay will approach that of a control containing no antithrombin. This was confirmed by adding 1·5 antithrombin units to 25 thrombin units in 5 ml. veronal buffer. After 18 hr. incubation at 37°, 15 thrombin units had been inactivated, and the thrombin decay rate was approximately equal to that of the control.

Effect of antithrombin on coagulation time of fibrinogen

The variation in coagulation time on recalcification of plasma has been used as a method of measuring antithrombin activity (Tanturi & Wetzel, 1949). This implies that antithrombin can

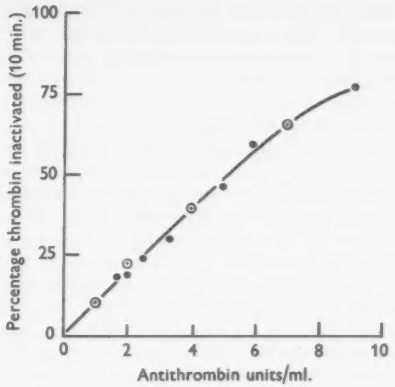

Fig. 3. Curve used for antithrombin estimation. ⊙, points derived from Fig. 1; ●, points derived from other experiments.

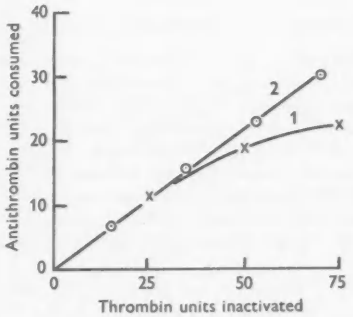

Fig. 4. Reduction in antithrombin activity caused by incubation with thrombin. Curve 1: twenty-five antithrombin units (0·5 ml. DFP), thrombin as shown; curve 2: fifty antithrombin units (1·0 ml. DFP), thrombin as shown.

Table 1. *Effect of antithrombin on coagulation time of fibrinogen*

In each case 2·0 ml. thrombin solution of appropriate concentration was added to give a total vol. of 8 ml.

Substrate	Thrombin in reaction mixture (units/ml.)			
	1·25	0·42	0·14	0·05
	Coagulation time (sec.)*			
1 ml. fibrinogen (1%, w/v, in veronal buffer) added to:				
5 ml. veronal buffer	14·0	31	79	185
5 ml. heated DFP	18·8	44	119	320
2·5 ml. heated DFP + 2·5 ml. unheated DFP	18·0	43	123	410
5 ml. unheated DFP	17·6	43	133	640

* Average of three measurements.

influence the rate of coagulation of fibrinogen by thrombin. The conditions under which this influence was significant were investigated.

Progressive dilutions of thrombin (5 units/ml.) were used to coagulate fibrinogen solutions containing various proportions of heated (10 min., 57°) and unheated DFP, the heated material being added to allow for the non-progressive prolongation of coagulation time already mentioned. The effect of active antithrombin in the unheated DFP is shown by Table 1. The immediate delaying action on coagulation, produced by both normal and heated DFP, was evident at all thrombin concentrations, whereas the additional effect of the progressive antithrombin was not significant until the coagulation time exceeded 100 sec., and not pronounced until coagulation times of the order of 400 sec. were involved. Measurement of the increase in coagulation time on addition of antithrombin to mixtures of thrombin and fibrinogen solutions is thus seen to be a very insensitive method of estimating antithrombin.

Concentration and purification of antithrombin

Identification of electrophoretic component containing antithrombin. Although the albumin fraction of serum is generally considered to be responsible for antithrombin activity (Lenggenhager, 1935; Quick, 1938; Astrup & Darling, 1942), the albumin studied by these authors was prepared by salt fractionation in each case, and was possibly not electrophoretically pure. To investigate the activity of pure albumin, two electrophoretic runs

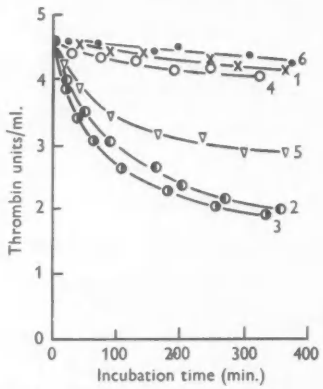

Fig. 5. Antithrombin activity of electrophoretically separated plasma fractions. Initial thrombin concentration 4·65 units/ml. in each case. Curve (1) 1 ml. albumin; (2) 1 ml. α-, β-, γ-globulin fraction; (3) 1 ml. albumin + α-globulin; (4) 1 ml. β- + γ-globulin; (5) 0·5 ml. albumin + 0·5 ml. α-, β-, γ-globulin; (6) control. Volume adjusted in each case with veronal buffer to 5 ml.

were carried out on DFP. In the first run a cut was made between the albumin and α-globulins on the ascending side, enabling albumin to be separated from this side of the U-tube and α-, β-, and γ-globulins from the descending side; while in the second run a cut was made between α- and β-globulins on the descending side, yielding β- and γ-globulins on this side and albumin and α-globulin on the ascending side. Inactivation curves were then measured on addition of each of these fractions to a solution of thrombin (Fig. 5).

The results show that electrophoretically pure albumin is not antithrombic; the antithrombin activity lies instead in the α-globulins, and follows this fraction on separation. No activity is exhibited by β- and γ-globulins, and a mixture of all the separated plasma components (curve 5) shows no more activity than that due to the α-globulins. The antithrombin activity of human plasma thus belongs to the α-globulin fraction alone.

A similar series of runs on fresh human serum showed that in serum as well as in DFP the antithrombin activity was associated with the α-globulin fraction.

Concentration of antithrombin by salt precipitation. Fractionation of DFP by precipitation with Na_2SO_4 and $(NH_4)_2SO_4$ at a variety of concentrations and pH values resulted in no concentration of antithrombin. The only conditions under which antithrombin was precipitated to any great extent were at high concentrations of ammonium sulphate (above 70% saturated) and neutral pH values, or moderate concentration of ammonium sulphate (50% saturated) and pH values below 4·5. Antithrombin thus appears to be among the more soluble globulins.

Concentration of antithrombin by ether precipitation. In the fractionation of human blood plasma by ether precipitation (Kekwick & Mackay, 1948, 1954), the fibrinogen, prothrombin-containing globulins, and a β- + γ-globulin fraction are successively removed from citrated plasma. The supernatant remaining contains albumin as well as the more soluble fractions of the α- and β-globulin. From this supernatant a precipitate was obtained by lowering the pH to 5·0 at −4°, the ether content remaining at 18% (v/v) and the ionic strength at 0·035. This precipitate contained a high proportion (30%) of α-globulin, and will subsequently be referred to as α-rich precipitate. The antithrombin activity of this precipitate per g. protein was 1·9 times that of DFP. Electrophoretic separation of the albumin + α-globulin fraction of this material in the flow-through U-tube increased the specific activity to 3·4 times that of DFP.

This partially purified antithrombin gave thrombin decay curves which yielded values for k_1 (first-order constant) which were within the range of

those measured from Figs. 1 and 2. The kinetics of reaction thus seemed the same as with unfractionated DFP.

Properties of antithrombin

pH *stability*. The pH stability of antithrombin was examined by adjusting the pH of 10 ml. samples of DFP with 0·2N-HCl or NaOH, and incubating for 4 hr. at 37°. The solutions were then neutralized and made up to a constant volume with 0·15M-NaCl. Samples of each mixture were drawn off for pH measurement during the incubation. The antithrombin activities remaining after neutralization were measured (Fig. 6). The antithrombin activity of DFP is stable under the conditions used between pH's 6 and 9·5, but is particularly susceptible to pH above 9·5. A second experiment, carried out in conjunction with the results described in the next section, showed that the antithrombin activity of α-rich precipitate was completely stable for 10 min. at 37° at pH values between 6 and 8·5.

Variation of activity with pH. Samples of the α-rich precipitate were incubated with solutions of thrombin at various pH values, and the thrombin inactivation was followed. As addition of a reaction mixture to the fibrinogen solution would change its pH, a modification of the usual method for estimating thrombin concentration was necessary to ensure that coagulation took place near pH 7·3. Samples (0·2 ml.) of the reaction mixtures were added to 1·0 ml. veronal buffer, pH 7·3, at 37°, and 0·2 ml. of this sixfold dilution was added to 0·8 ml. fibrinogen solution in the usual way, so that coagulation took place under the standard conditions between pH's 7·2 and 7·4. Control solutions of thrombin alone were also incubated at a series of pH values to determine how much of the thrombin inactivation occurring in the reaction mixtures was due to pH alone. The curves of Fig. 7 are corrected for this effect.

The previous section on pH stability indicates that the shape of the activity curve (Fig. 7) is not due to inactivation of antithrombin at pH values on either side of the point of maximum activity, but rather to a true pH optimum in the antithrombin activity in the vicinity of pH 7·4.

Temperature stability. In order to examine the temperature stability of antithrombin, 10 ml. samples of α-rich precipitate, dissolved in veronal buffer, pH 7·3, were heated to various temperatures for 10 min. in thin-walled glass tubes, chilled in cold water, and the antithrombin activities measured.

The results (Table 2) indicate that antithrombin is comparatively stable up to 50°, but at 60° it is rapidly inactivated. At 37°, antithrombin loses no appreciable activity in 24 hr.

Influence of temperature on activity. Identical mixtures of thrombin and α-rich precipitate were incubated at various temperatures and the course of the reaction followed by adding 0·2 ml. samples of the mixture to 1·0 ml. veronal buffer at 37°, then

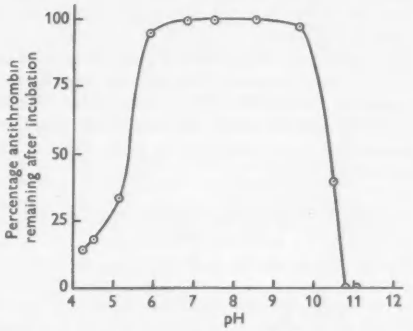

Fig. 6. Stability of antithrombin at different values of pH; DFP incubated at the values of pH shown for 4 hr. at 37°.

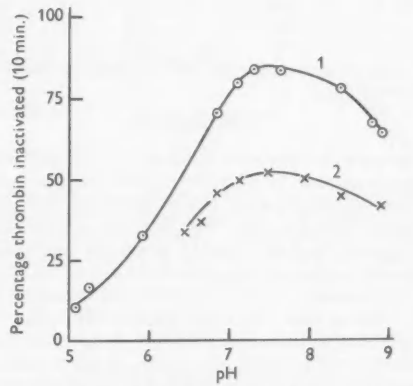

Fig. 7. Variation of antithrombin activity with pH. Curve 1: 1·0 ml. thrombin (50 units/ml.) + 0·5 ml. α-rich precipitate + 0·15N-NaOH or 0·15N-HCl; volume adjusted to 5 ml. with veronal buffer; curve 2: as above, except that 0·25 ml. α-rich precipitate used in place of 0·5 ml.

Table 2. *Temperature stability of antithrombin*

Temperature of incubation (°)	Antithrombin remaining after 10 min. incubation (units/ml.)	Antithrombin inactivated (%)
35	50	0
40	49	2
45	51	−2
50	50	0
55	41	18
60	5	90
65	0	100

using 0·2 ml. of this sixfold dilution to produce coagulation of 0·8 ml. fibrinogen solution in the usual way. This method avoided alteration of the temperature of the coagulation mixture, so enabling the normal calibration curve to be used. Control samples of thrombin alone were heated in a similar manner in order to allow for the thermal inactivation of thrombin.

The results of Table 3 show that the percentage thrombin inactivated reaches a broad maximum in the region of 35–40°. As antithrombin itself is completely stable under the conditions used, this represents a true temperature optimum for antithrombin activity.

Table 3. *Variation of antithrombin activity with temperature*

Temperature (°)	Thrombin inactivated* in 10 min. (%)
25	39
30	44
35	49
40	49
45	46
50	43

* Inactivation corrected for spontaneous decay of thrombin.

DISCUSSION

The main problem with which this investigation has been concerned is that of the rate and the extent of the inactivation of human thrombin by human plasma components. The normal antithrombin action of blood has been shown to be progressive, the rate of thrombin inactivation depending on the concentration of both thrombin and antithrombin. In view of this, the most satisfactory method of measuring antithrombin activity is in terms of the velocity of thrombin inactivation. A direct measurement from the number of thrombin units inactivated is not permissible, although this method is used by Astrup & Darling (1942) in estimating antithrombin of ox blood. Further, it is clear from Table 1 that the prolongation of coagulation time produced by addition of antithrombin to a mixture of thrombin and fibrinogen solutions is insensitive even to large changes in antithrombin concentration if thrombin is present in other than very low concentration. This observation is in accord with the evidence that a rise in the antithrombin content of blood does not produce a prolongation of coagulation time (Volkert, 1942; Koller & Fritschy, 1947).

It does not seem necessary to postulate a competitive partition of thrombin between fibrinogen and antithrombin to account for the fact that antithrombin does not act as an anticoagulant, although this idea has been suggested (Quick, 1938). The fact that antithrombin is not immediate in its action is adequate to explain why there is no significant delay of coagulation on increasing the concentration of antithrombin when coagulation times of less than 100 sec. are involved. The action of thrombin on fibrinogen certainly precedes the appearance of the clot, so that the removal of thrombin from the system by antithrombin would have to be extremely rapid to impose a delay on this early phase of the action on fibrinogen.

The non-progressive delaying action of defibrinated plasma on coagulation has not been thoroughly investigated here. The delay is not a non-specific colloid effect, for human γ-globulin solution containing the same weight of protein/unit vol. as defibrinated plasma does not produce it either before or after heating; and gum acacia solutions actually cause a considerable increase in the rate of coagulation. The delay may well be due to a reversible adsorption of thrombin on to albumin, as was suggested long ago by Landsberg (1913) and more recently by Gerendas (1948). It is, however, quite distinct from the true progressive antithrombin action, and a study of antithrombin must make use of methods which distinguish between the two effects.

Whatever may be the nature of the reaction causing the progressive decay of thrombin, it has been shown that the antithrombin responsible occurs in the α-globulin fraction of both serum and defibrinated plasma, not in the albumin as previously supposed. Fractionation of defibrinated plasma by salt precipitation has not been successful in separating this antithrombic α-globulin from albumin, and ether precipitation has achieved only a partial separation. The problem of purifying antithrombin is the difficult one of removing a small amount of active material from a large excess of contaminant, in this case albumin, with closely similar physical properties.

SUMMARY

1. The antithrombin action of defibrinated human plasma on human thrombin has been studied.

2. A method of estimating antithrombin based on the rate of thrombin inactivation has been developed.

3. A partial purification of antithrombin has been achieved by ether fractionation of plasma; the active fraction contained 30% α-globulin.

4. α-Globulin isolated electrophoretically from this fraction was shown to carry the antithrombin activity.

5. Antithrombin is stable at 37° between pH 6·0 and 8·5 and shows an optimum activity at pH 7·3.

6. At pH 7·3 antithrombin is comparatively

stable up to 50°. The temperature optimum for antithrombin activity is between 35 and 40°.

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The Antithrombin Activity of Heparin

By J. W. LYTTLETON*

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Since its discovery by MacLean (1916) heparin has been recognized as the most important naturally occurring anticoagulant. It seems well established that its action is twofold; it inhibits the transformation of prothrombin to thrombin when accompanied by a cofactor present in plasma (Brinkhous, Smith, Warner & Seegers, 1939), and it inhibits the action of thrombin on fibrinogen. It is this second action of heparin as an antithrombin that is studied here.

MATERIALS AND METHODS

'Heparin Vitrum', Stockholm, with an activity of 100 units/mg. dry weight (Murray & Best, 1938) was dissolved in small amounts to give solutions of 100 units/ml. in veronal buffer, pH 7·3, $I = 0{\cdot}15$ (Michaelis, 1930), and freshly diluted as required. Toluidine blue (British Drug Houses Ltd.) was dissolved in veronal buffer to give a solution of 50 mg./ml. Thrombin, fibrinogen, and defibrinated plasma were the same as those described in the previous paper (Lyttleton, 1954).

Measurement of antithrombin activity of heparin. To study the antithrombin effect of heparin, the type of experiment was the same as described in the previous paper (Lyttleton, 1954). Thrombin was incubated at 37° with heparin and any other material being studied, and the decay in thrombin activity was followed by allowing samples withdrawn after various time intervals to coagulate a standard fibrinogen solution at 37°.

RESULTS

Action of heparin on thrombin

Samples (5·0 ml.) of thrombin (6·7 units/ml.) were incubated with various concentrations of heparin at 37°. Some of the curves thus obtained are shown in Fig. 1.

It is plain that heparin exerts two different effects on this thrombin preparation: (i) a slow progressive inactivation, predominant at low concentrations of heparin, and (ii) an immediate drop in thrombin activity, predominant at concentrations of heparin above 1·0 unit/ml.

From 0·05 to 0·5 unit/ml. of heparin, the rate of progressive inactivation of thrombin remains almost independent of heparin concentration, though this finding is not illustrated in Fig. 1. At 0·5 unit/ml. of heparin an immediate decrease in thrombin activity was observed, and further increase in heparin concentration caused a greater immediate decrease in

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thrombin activity, whilst the subsequent rate of progressive inactivation was reduced. Thus after periods of 100 min. those solutions containing stronger heparin (Fig. 1, curves 5 and 6) had more thrombin activity than those with weaker heparin (curves 2 and 3), a reversal of the position shown immediately after mixing. These two different actions were subjected to further study.

Immediate reduction in thrombin activity caused by larger amounts of heparin. This section deals with the effect on thrombin of heparin in concentrations greater than 2 units/ml. The magnitude of the apparent immediate thrombin inactivation does not increase linearly with increasing concentration of heparin, but approaches a limiting value as indicated in Fig. 1.

Provided that the coagulating mixture contained the same concentration of heparin, the same prolonged value of the coagulation time was found whether the heparin was originally added to the fibrinogen or to the thrombin. The effect was therefore a rapid one, occurring in the few seconds before coagulation took place.

Progressive dilutions of two thrombin samples, one containing no heparin and the other 10 units/ml., gave coagulation times which coincided when the dilution had reduced the heparin concentration to 0·2 unit/ml., indicating that the effect was reversible on dilution. This reversibility was also indicated by experiments with toluidine blue which removes heparin by combining with it (Jorpes, 1936). Unfortunately toluidine blue produces an immediate apparent increase in thrombin activity followed by a progressive decay, so conditions must be carefully controlled to produce a significant result.

Increase of the ionic strength of the solution containing thrombin from 0·15 to 0·5 led to a reduced drop in apparent thrombin strength when heparin was added. A new calibration of coagulation time against thrombin units was necessary to determine the effect due to the change in ionic strength alone. It was shown that at $I = 0{\cdot}15$, 8 heparin units/ml. reduced the activity of a thrombin solution of 12 units/ml. by 45%, while at $I = 0{\cdot}5$ the reduction was only 10%.

These results are all consistent with the hypothesis that thrombin will combine with heparin as a result of electrostatic attraction between the molecules to form a reversible thrombin–heparin complex which has less activity than the uncombined thrombin. This complex dissociates on dilution, increasing the ionic strength, or removing heparin from the system.

Evidence for the existence of the complex was obtained by carrying out an electrophoretic separation on two samples of thrombin, one containing no heparin, and the other containing 13 units/ml. The electrophoretic patterns produced were identical in the two cases and exhibited three components. Since their mobilities were too close to permit satisfactory separation, a sharply defined pigmented layer coinciding with the middle peak was chosen as the point at which to cut the pattern. The thrombin activity of the leading (anode) and trailing (cathode) sections of the separated fractions was examined under the usual conditions. The results are given in Table 1. No attempt was made to remove heparin from the fractions obtained by the second separation. It was presumably present at least in the leading fraction and would exhibit its usual delaying effect on coagulation carried out with this fraction. Despite this, the addition of heparin caused the transfer of a large part of the thrombin activity from the trailing to the leading fraction. As all other factors were kept constant during these

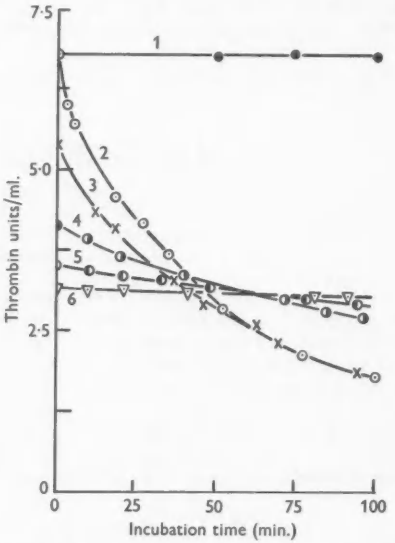

Fig. 1. Action of heparin on thrombin. Constant initial thrombin concentration (6·7 units/ml.). Heparin concns. (units/ml.): curve (1) 0·0; (2) 0·073; (3) 0·66; (4) 4·0; (5) 10·0; (6) 40·0. Volumes adjusted with veronal buffer.

Table 1. *Thrombin activity of electrophoretically separated fractions*

See text for experimental details.

	Coagulation times (sec.)	
Material	Leading fraction (anode)	Trailing fraction (cathode)
Thrombin alone	210	9
Thrombin + heparin	13	19

two runs, the increased mobility of the thrombin must be due to the action of heparin, which is consistent with the suggestion that a complex is formed between the two substances which, because of the high negative charge on the heparin, migrates more rapidly than thrombin alone.

Progressive decay of thrombin due to smaller amounts of heparin. This section deals with the action on thrombin of heparin in concentrations below 0·5 unit/ml. The rate of the progressive decay reaction is not appreciably influenced by the concentration of heparin until this is sufficiently great to produce considerable heparin–thrombin complex formation. Fig. 1 indicates that beyond this point the rate of progressive decay of thrombin decreases, as if the complex formation exerted a stabilizing influence on the thrombin. The loss in thrombin activity produced by heparin as a result of this decay is not reversible on removal of the heparin; this was demonstrated by addition of toluidine blue at various intervals during the progress of the reaction, which did not restore the thrombin activity to the initial level.

The fact that fibrin clots produced by thrombin mixtures containing heparin would lyse considerably more rapidly at 37° (1–2 hr.) than those which were free from heparin (12–24 hr.) suggested that the cause of the thrombin decay might be due either to activation of plasminogen in the thrombin preparation by heparin, or to an increase in susceptibility of thrombin to plasmin present. This was tested by adding to the thrombin–heparin system fractions of human serum containing plasminogen and plasmin respectively. These were prepared by diluting serum to 20 vol. with distilled water and lowering the pH to 5·3 with 0·5N acetic acid. The resulting precipitate, redissolved to 1/10 original volume in veronal buffer and neutralized, possessed only very slight antithrombin activity, and is referred to as serum euglobulin. On incubation with chloroform at 37° for 3 hr. the plasminogen contained in this fraction became activated to plasmin.

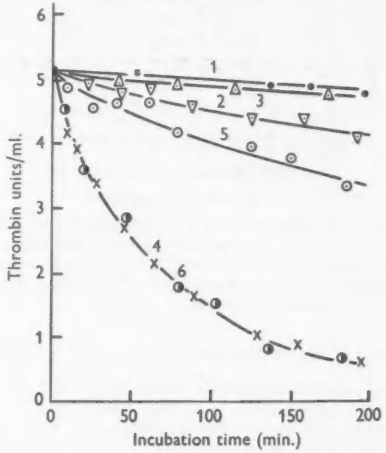

Fig. 2. Effect of serum euglobulins on action of heparin on thrombin. Initial thrombin concn. 5·1 units/ml. Volume of all mixtures made up to 5 ml. with veronal buffer. Curve (1) control; (2) 0·5 ml. euglobulin soln.; (3) 0·5 ml. euglobulin soln. ($CHCl_3$ treated); (4) 0·2 heparin unit/ml.; (5) 0·2 heparin unit/ml. + 0·5 ml. euglobulin soln.; (6) 0·2 heparin unit/ml. + 0·5 ml. euglobulin soln. ($CHCl_3$ treated).

The effect of adding euglobulin before and after treatment with chloroform, that is plasminogen or plasmin, to the heparin–thrombin system is shown in Fig. 2. Whereas the plasmin fraction had no effect on the action of heparin on thrombin, the plasminogen fraction produced, unexpectedly, considerable inhibition of the thrombin decay. It seems therefore that heparin does not act on thrombin through the plasma proteolytic enzyme plasmin. The component of the euglobulin fraction which is responsible for the inhibition of the thrombin inactivation has not been completely identified, but electrophoretic fractionation of the euglobulin showed that the inhibitory activity lay in the more mobile of the two components observed at pH 8.

Action of defibrinated plasma (DFP) on thrombin in the presence of heparin

Immediate effect (varying heparin concentration; constant DFP and initial thrombin). The curves of Fig. 3 (*a*) and (*b*) show the effect of varying the heparin concentration in the system thrombin–DFP–heparin. In group (*a*) even at very low concentrations of heparin there is a rapid fall in thrombin activity to a definite level, the time of fall increasing from less than 30 sec. at heparin concentration above 0·1 unit/ml. to approx. 3 min. at a concentration of 0·025 unit/ml. ($0·25 \times 10^{-6}$ g./ml.)

Group (*b*) indicates that as the concentration of heparin increases beyond this range the initial drop in thrombin activity increases, because the effect of heparin–thrombin complex formation is added to that of DFP in the presence of heparin.

To study the immediate action of DFP on thrombin in the presence of heparin, it is therefore advisable to use a heparin concentration of about 0·1 unit/ml. In this region the initial drop in thrombin activity is rapid, and there is no appreciable heparin–thrombin complex formation.

Immediate effect (varying DFP concentration; constant heparin and initial thrombin). Mixtures containing 10 units/ml. thrombin, 0·1 unit/ml. heparin and varying amounts of DFP were assayed for thrombin activity every 2 min. during 10 min., and the data obtained extrapolated to zero time to

give a measure of the thrombin inactivated by the immediate action of DFP in the presence of heparin. In Fig. 4, curve 1, the thrombin inactivated at zero time is plotted against concentration of DFP, the curve obtained approaching linearity. Provided that the concentration of heparin is 0·2 unit/ml. or less, an almost linear relationship exists between the concentration of DFP in a mixture and the amount of thrombin inactivated in the first 30 sec.

If heparin at higher concentrations is used in this experiment, two control solutions are necessary, one containing thrombin alone (*A*), and the other containing thrombin plus heparin (*B*). On adding DFP to the thrombin and heparin, a drop in thrombin activity occurred; and if the apparent thrombin inactivation relative to the control solution (*B*) was plotted against the amount of DFP added, a curve such as curve 2, Fig. 4, was obtained, the slope becoming further removed from that of curve 1 as the heparin concentration was increased.

This departure from curve 1 may be explained by assuming that relative to the control (*A*) the amount of thrombin inactivated by DFP, even in the presence of strong heparin, is the same as in curve 1, and that the remaining thrombin undergoes complex formation with heparin to give a further reduction in thrombin activity in the same ratio as that between controls (*A*) and (*B*), the decrease in activity for curve 2 being 40%. The thrombin activities predicted on this assumption agree with those measured.

Immediate effect (varying thrombin concentration; constant heparin and DFP). The immediate thrombin inactivation produced in these circumstances is illustrated in Table 2, which shows the

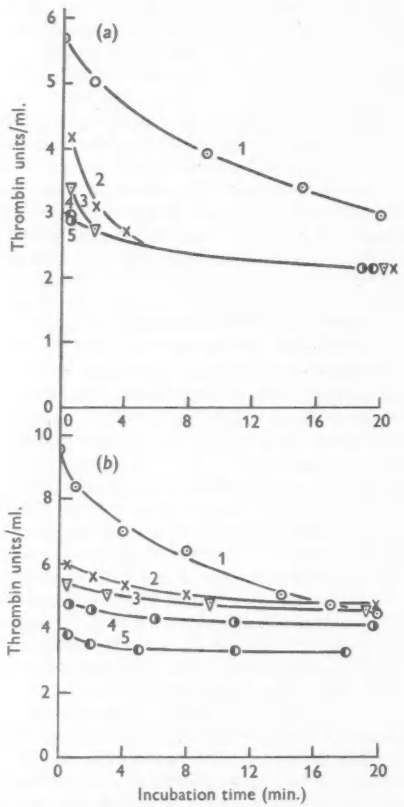

Fig. 3. Immediate action of DFP on thrombin in presence of heparin. (*a*) Initial thrombin concn. 5·7 units/ml.; DFP concn. 0·05 ml./ml. soln.; solution volume 5 ml.; heparin concns. (units/ml.): curve (1) 0; (2) 0·025; (3) 0·05; (4) 0·1; (5) 0·2. (*b*) Initial thrombin concn. 9·5 units/ml.; DFP concn. 0·08 ml./ml. soln.; solution volume 5 ml.; heparin concns. (units/ml.): curve (1) 0; (2) 0·11; (3) 0·33; (4) 1·0; (5) 3·0.

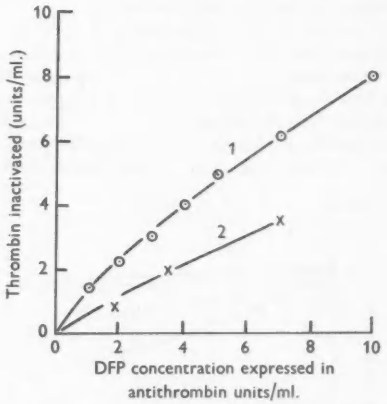

Fig. 4. Immediate action of DFP on thrombin in presence of heparin. Curve 1: heparin concn. 0·1 unit/ml.; initial thrombin concn. 10 units/ml. Curve 2: heparin concn. 3·0 units/ml.; initial thrombin concn. 10 units/ml. Volumes adjusted with veronal buffer.

Table 2. *Thrombin inactivation by DFP and heparin*

5 ml. samples of solutions containing 0·1 heparin unit/ml., 5·0 antithrombin units/ml. (DFP), and varying thrombin units/ml., incubated at 37°.

Initial thrombin concn. (units/ml.)	Thrombin inactivated, in 30 sec. (units/ml.)
15·6	5·0
10·7	5·1
8·0	5·0
5·7	4·8
2·8	2·8

effect of adding heparin and DFP in constant amounts to a series of thrombin solutions. The amount of thrombin inactivated by the initial rapid reaction is apparently independent of the initial thrombin concentration.

Subsequent progressive decay of thrombin activity. After the immediate inactivation considered above has taken place, further change in thrombin activity is influenced by at least two factors: the action of the antithrombin remaining in the DFP, and the progressive action of the heparin itself. Fig. 5 shows examples of thrombin decay curves obtained when the amount of heparin present in the system was varied. The thrombin preparation used in these experiments was less susceptible to the progressive action of heparin alone than the thrombin used in the experiments of Fig. 1, as shown by the reduced rate of decay in the corresponding curves. The reason for this reduced susceptibility is not known, but the thrombin preparation may have contained a larger amount of the heparin-inhibiting globulin. The use of this particular material reduced the decay due to heparin alone, and allowed the decay due to the remaining antithrombin to become more prominent.

The immediate change in the thrombin activity as the heparin concentration is varied is of the same type as that already illustrated in Figs. 1 and 3. The rate of the subsequent inactivation in the presence of DFP then decreases as the heparin concentration increases, so that after a sufficient time (150 min.) has elapsed, the thrombin concentrations in curves 6–8 (Fig. 5) are in reverse order to those measured earlier (5 min.). It is probable that as the heparin concentration increases, heparin–thrombin complex formation occurs to an increasing extent, and this complex may well be less sensitive than free thrombin to the action of any antithrombin remaining in the system. As the free thrombin in equilibrium with the complex is inactivated, the complex will presumably dissociate to liberate more thrombin, giving an apparently reduced decay rate of thrombin activity.

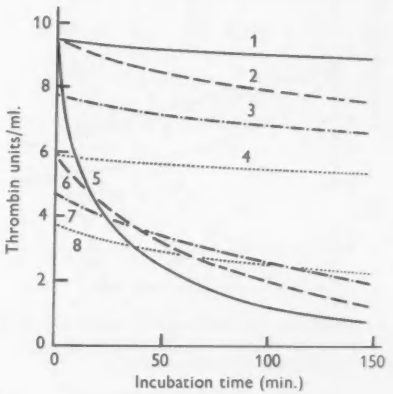

Fig. 5. Inactivation of thrombin by DFP in presence of heparin. Constant initial thrombin concn. 9·5 units/ml. Curves 1–4, no DFP; curves 5–8 DFP added to give 3·5 antithrombin units/ml. Heparin concns. (units/ml.): curves (1) and (5) 0; (2) and (6) 0·1; (3) and (7) 1·0; (4) and (8) 3·0. Volumes adjusted with veronal buffer.

It is also possible that the antithrombin activity of the mixture may be reduced by the reaction resulting in the immediate inactivation of thrombin. The role of antithrombin in this process will be discussed more fully later, but it will be noticed that after the first 10 min. the mixture containing DFP but no heparin (Fig. 5, curve 5) inactivates thrombin at a greater rate than the mixture containing DFP and 0·1 heparin unit/ml. (curve 6), despite the fact that heparin itself contributes to the progressive thrombin decay. This indicates that at least a part of the antithrombin activity of the DFP has been removed by the initial rapid reaction in the presence of heparin.

Addition of toluidine blue to the system to remove the heparin would not simplify matters by allowing the decay due to remaining antithrombin action alone to be studied, as the toluidine blue itself produces a decay in thrombin activity.

Heparin cofactor

The results suggest that the immediate inactivation of thrombin is due to a combination, in the presence of heparin, between thrombin and some factor present in DFP; this factor will be referred to as 'heparin cofactor'.

An experiment carried out by mixing thrombin, heparin, DFP, and toluidine blue in different sequences showed that unless thrombin, heparin, and DFP were present simultaneously no inactivating combination took place. If heparin, after a short incubation with either thrombin or DFP, was removed by the addition of toluidine blue, the subsequent addition of DFP or thrombin resulted in no immediate drop in thrombin activity. Either the heparin is bound irreversibly in the thrombin–cofactor complex, or it acts merely as a catalyst in the formation of this complex. This experiment did not decide between these possibilities.

Estimation of heparin cofactor. On the basis of Fig. 4, which shows that a definite amount of DFP in the presence of a low concentration of heparin (0·1 unit/ml.) will produce immediate inactivation of a definite amount of thrombin, a quantitative estimation of heparin cofactor may be made by measuring thrombin inactivation under these conditions. By analogy with the antithrombin unit, 1 ml. DFP is defined as containing 50 units of cofactor.

Interrelation of antithrombin and heparin cofactor

Heparin cofactor and antithrombin have been considered to be distinct so far in this paper, but it is possible that they may be identical, a view proposed by Quick (1938) and Seegers (1942), and that heparin catalyses the action between thrombin and antithrombin. Evidence will now be considered leading to a choice between these alternatives.

Qualitative aspects. In general the cofactor follows antithrombin when DFP is fractionated by precipitation or electrophoretic separation. Experiments similar to those described for antithrombin (Lyttleton, 1954) established that heparin cofactor is an α-globulin, and that albumin, β- and γ-globulin are inactive. Electrophoretically separated albumin + α-globulin fractions from DFP showed the same antithrombin : cofactor ratio as unfractionated DFP. However, the α-globulin-rich precipitate prepared by fractionation with ether (Lyttleton, 1954) had a cofactor activity 2·5 times that of DFP, some 30% greater than the relative antithrombin activity (1·92).

Any reaction which consumes one factor alters the other. If the antithrombin content of DFP is reduced by incubating with thrombin, the amount of cofactor remaining in the DFP is simultaneously decreased. Also as already mentioned (Fig. 5) the immediate reaction of cofactor in DFP in the presence of heparin seems to reduce the amount of antithrombin remaining. These two effects are shown more directly by the following experiments. In the first, heparin was added to a series of thrombin–DFP mixtures after different periods of incubation at 37°. During the interval, before the addition of heparin, the usual progressive thrombin decay took place; when heparin was added a rapid fall in thrombin activity occurred, its extent depending on the amount of cofactor remaining in the solution. It is evident from curves 4–7 in Fig. 6 that as the thrombin–antithrombin reaction proceeded, and antithrombin was used up, the amount of heparin cofactor available also decreased.

In the second experiment the rate of decay of three thrombin solutions of 5·5 units/ml. was studied (Fig. 7). One of these solutions contained DFP, another contained heparin, and the third was formed from a stronger thrombin solution to which both heparin and DFP had been added, the resulting rapid fall in thrombin activity due to combination with heparin cofactor leaving approximately 5·5 units/ml., together with the remaining components of DFP. The rate of decay of these solutions indicated that removal of cofactor from DFP reduced its ability to act as a progressive antithrombin, curve 2 decaying less rapidly than curve 4.

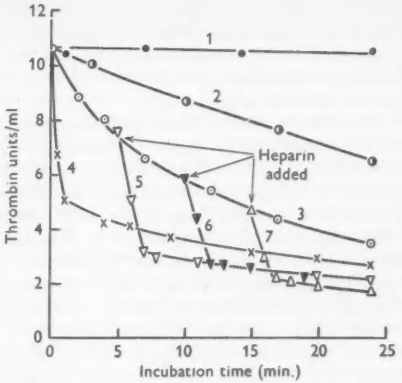

Fig. 6. Reduction of available heparin cofactor during inactivation of thrombin by DFP. All solutions contained 1 ml. thrombin (53 units/ml.) and were made up to 5 ml. with veronal buffer. Curve (1) control; (2) 0·05 ml. heparin (5 units/ml.); (3) 0·5 ml. DFP; (4) 0·5 ml. DFP + 0·05 ml. heparin (added after 0 min.); (5) 0·5 ml. DFP + 0·05 ml. heparin (added after 5 min.); (6) 0·5 ml. DFP + 0·05 ml. heparin (added after 10 min.); (7) 0·5 ml. DFP + 0·05 ml. heparin (added after 15 min.).

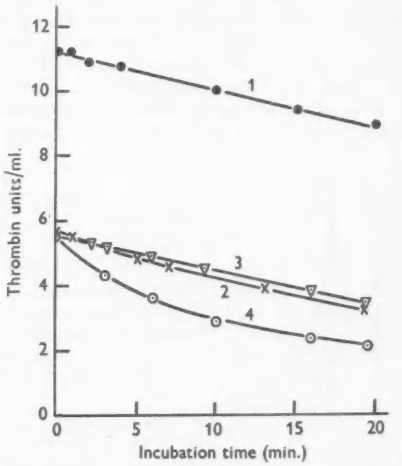

Fig. 7. Reduction of antithrombic action of DFP by removal of heparin cofactor. The curves correspond to the following mixtures (volumes in ml.):

	Thrombin (56 units/ml.)	Veronal buffer	Heparin (0·5 unit/ml.)	DFP
(1)	1·0	3·5	0·5	0
(2)	1·0	3·0	0·5	0·5
(3)	0·5	4·0	0·5	0
(4)	0·5	4·0	0	0·5

Further evidence suggesting that the normal antithrombin activity of DFP and the action in the presence of heparin may be related lies in the fact that toluidine blue (an anti-heparin) reduces the rate of thrombin inactivation by normal antithrombin when added to the system DFP–thrombin. A quantitative measure of the effect would require a more complete study of the inactivation of thrombin caused by toluidine blue alone, but if this is disregarded, 0·1 mg. toluidine blue/ml. is found to reduce the apparent antithrombin activity of DFP by 35%.

Quantitative aspects. It is difficult, because of the direct action of heparin on thrombin, to estimate the amount of antithrombin remaining in a DFP–thrombin–heparin mixture when the cofactor has been used up; but the converse estimation of the cofactor remaining after removal of antithrombin may readily be carried out. 0·5 ml. DFP was incubated with 0, 25, 50, and 75 units of thrombin for 18 hr. at room temperature, after which time less than 0·1 unit thrombin remained. The amounts of antithrombin and heparin cofactor remaining in each solution were determined (Table 3).

Table 3. *Inactivation of antithrombin and heparin cofactor on incubation with thrombin*

Thrombin units inactivated	Antithrombin units remaining	Heparin cofactor units remaining
0	26	23
25	15	7
50	9·5	3
75	5	0

Table 4. *Progressive decrease in antithrombin and heparin cofactor during inactivation of thrombin*

Time (min.)	Antithrombin units present	Heparin cofactor units present
0	5·0	5·2
5	3·8	4·2
10	3·3	3·2
15	3·1	1·7

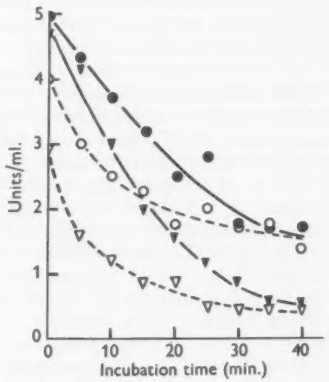

Fig. 8. Decay of antithrombin and heparin cofactor. Initial activity in all cases 5 units/ml. ● and ○, antithrombin; ▼ and ▽, heparin cofactor. ●, ▼, decay at pH 7, 57°; ○, ▽, decay at pH 4·5, 37°.

A similar type of analysis is illustrated in Fig. 6. By measuring the percentage thrombin inactivation during successive 10 min. intervals in curve 3, the variation in antithrombin content with time may be calculated, while from the discontinuities in curves 4–7 the amount of heparin cofactor remaining after a given time may be calculated. The results obtained in this way are given in Table 4.

These results show that antithrombin and heparin cofactor cannot be identical: a view supported by a study of the rate of decay of the two factors.

Decay of antithrombin and heparin cofactor. This was studied under two sets of conditions. In the first case, DFP was heated to 57°, and 2 ml. samples were withdrawn and mixed with equal volumes of cold veronal buffer after successive 5 min. intervals. The antithrombin and cofactor contents were later measured. In the second case, DFP at 37° was acidified to pH 4·75 with 0·2N-HCl. Samples were withdrawn in the same way, and added to buffer to which enough NaOH had been added to produce a pH of 7·3 on mixing. The two activities were later measured. Both results are shown in Fig. 8. It is clear that the cofactor is more labile than the antithrombin in both the experiments, and from all these considerations it must be concluded that, though antithrombin and heparin cofactor are closely related, they cannot be identical.

DISCUSSION

It has been shown that heparin has a direct effect on thrombin, independent of its more important antithrombic action in the presence of a plasma cofactor. At heparin concentrations above 2·0 units/ml. there is good evidence that heparin–thrombin complex formation occurs, the thrombin activity being thereby reduced. The mechanism of the progressive inactivation produced by lower concentrations of heparin has not yet been established.

In the presence of heparin concentrations of more than 0·05 unit/ml. the action of defibrinated plasma on thrombin changes from a progressive to an immediate inactivation, the extent of which depends only on the concentration of defibrinated plasma. The simplest hypothesis to explain this is that of Seegers (1942) who suggested that antithrombin could combine only with a definite amount of thrombin, and that the action of heparin was merely catalytic. This implies that antithrombin and heparin cofactor are identical. This hypothesis, however, does not fully explain the observed facts.

Defibrinated plasma alone can ultimately inactivate considerably more thrombin than is immediately inactivated in the presence of heparin (Fig. 5) so that, even if heparin does catalyse a combination between thrombin and antithrombin, it does not utilize to the full the capacity of defibrinated plasma to inactivate thrombin. After reacting with thrombin in the presence of heparin, defibrinated plasma is capable of inactivating still more thrombin though its potency is considerably reduced. Increasing the heparin concentration does not result in an increase in the immediate thrombin inactivation, apart from the effect due to heparin alone, so the remaining progressive antithrombin action cannot be converted into an immediate one by the action of heparin.

It seems necessary to postulate two processes which take place during inactivation of thrombin by defibrinated plasma: (i) an inhibitory combination, catalysed by heparin; (ii) a progressive inactivation, not catalysed by heparin. The first of these reactions is due to heparin cofactor, and leads to immediate thrombin inactivation in the presence of heparin of concentrations above 0·05 unit/ml. It has been shown, however, that if the heparin concentration is decreased below this, the rate of the initial inactivation of thrombin is decreased (Fig. 3*a*).

It has been claimed (Jaques, Monkhouse & Stewart, 1949) that human blood normally contains heparin in concentrations of the order of 0·0001 mg./ml. or 0·01 unit/ml. It is probable that in the presence of this very small amount of heparin the first reaction will take place even more slowly, at a rate comparable with the action of progressive antithrombin. Addition of heparin to the system accelerates a process that normally takes some minutes, thereby producing rapid inactivation. As it is not known whether this substance would react in the complete absence of heparin, it will still be referred to as heparin cofactor.

The second reaction postulated, a progressive inactivation not catalysed by heparin, may be due either to a second inhibitory combination or to an enzymic inactivation of thrombin which is ultimately slowed down in some way, possibly due to inhibition by end products of the reaction. This is evident from the results obtained on incubating defibrinated plasma with an excess of thrombin for a long period, when progressive thrombin inactivation ultimately ceases. For convenience the substance involved in this reaction will be called thrombin inactivator.

If this hypothesis is accepted, it is clear that when a measurement is made on the 'antithrombin' of defibrinated plasma both reactions are involved. When, however, the heparin cofactor is measured by immediate thrombin inactivation, it is only the first reaction involving inhibitory combination that is concerned. In the light of this, many of the observed facts in this and the previous paper (Lyttleton, 1954) may be explained.

The decrease in value of the first-order reaction constants with time in the reaction between antithrombin and thrombin may be ascribed to the fact that both the heparin cofactor and the thrombin inactivator are initially involved; but after a time the cofactor is saturated by combination with thrombin and the inactivator continues its action alone. The differences in stability of heparin cofactor and antithrombin are due to the fact that the antithrombin consists of both the cofactor and thrombin inactivator of which the former is the more rapidly destroyed. The reduction in antithrombin produced by incubation of defibrinated plasma with thrombin again involves both factors, and the more readily saturated heparin cofactor will be removed first from the system as the amount of thrombin incubated is increased, leaving mainly thrombin inactivator. Evidence of this is shown in the analysis of Fig. 6 already quoted (Table 4). The antithrombin and heparin cofactor both fall rapidly at first, but the antithrombin later falls more slowly. This is consistent with an antithrombin consisting of both cofactor (more rapidly removed) and thrombin inactivator, the action of which becomes more prominent as the cofactor disappears.

Several criticisms may be levelled at this hypothesis. In the first place, it has been shown that, even after extensive fractionation of defibrinated plasma involving ether precipitation followed by electrophoretic separation, the thrombin inactivation produced seems to follow the same kinetic course of reaction as that produced by unfractionated defibrinated plasma. If the above theory is correct, then both the heparin cofactor and the thrombin inactivator, as well as a trace of heparin to act as catalyst, must be present in approximately the same proportions before and after fractionation. In view of the fact that no electrophoretic component other than α-globulin showed any antithrombin activity, both factors must belong to this plasma component. It is possible, therefore, that they possess sufficiently similar physical properties to follow one another on fractionation. Heparin may act primarily through the strong electric charge on its molecule (Jorpes, 1946), since it is known to combine with plasma proteins (Chargaff, Ziff & Moore, 1941). It is consequently quite possible that heparin follows its cofactor on fractionation by virtue of some form of combination.

In the second place, it seems from Fig. 5 that more thrombin can be inactivated by defibrinated plasma alone, than when heparin is added to the system. The hypothesis requires the first reaction of inhibitory combination to be catalysed by heparin, and the second reaction to proceed unchanged, the

total amount of thrombin inactivated being unchanged. To explain the discrepancy it must be pointed out that the rate of action of thrombin inactivator depends on the thrombin concentration. In the absence of heparin (Fig. 5, curve 5), the thrombin drops progressively from its initial concentration, and in the earlier stages of the reaction the rate of the decay due to the inactivator is maximal. When heparin is present in low concentration (curve 6) the thrombin concentration immediately drops to a lower level and consequently the action of the inactivator is never as rapid as in the first case, with the result that the thrombin activity remains above that for the defibrinated plasma alone after a certain period. In other words, when the two reactions are simultaneous, in the absence of added heparin, the inactivator is able to work at a higher rate than when they are consecutive.

The behaviour of the other curves in Fig. 5 can be ascribed to heparin–thrombin complex formation, if it is assumed that this complex is not susceptible to attack from the thrombin inactivator.

The hypothesis cannot, however, be taken as having been established. It is practically impossible to confirm it with the materials at present available and a more complete isolation of the two postulated substances is necessary. In the absence of such isolation the thrombin inactivator could presumably be investigated by using up all the cofactor by adding heparin, then studying the ensuing decay due to the inactivator alone; but heparin itself produces thrombin decay, at least in the preparations used here. It should similarly be possible to study the thrombin inactivator alone by removing all the heparin from the system with toluidine blue, thus rendering the cofactor inactive; but toluidine blue itself modifies the coagulant action of thrombin and produces thrombin decay. Nevertheless, it has already been mentioned that in the presence of toluidine blue the antithrombin action of defibrinated plasma is reduced, and though this observation is not capable of quantitative interpretation it is in support of the proposed theory.

The situation then is that there is no evidence directly opposed to the hypothesis, and no clear-cut evidence which can be considered to establish it; but it can certainly be used with success in interpreting the results obtained here.

The ideas put forward here are similar to those of Gerendas (1948), who considers the action of antithrombin to consist of two parts, an immediate absorption followed by a progressive inactivation, considered to be enzymic. Examination of his work, however, shows that the immediate absorption which he considers, is the non-progressive effect of strong solutions of defibrinated plasma in delaying coagulation, which persists after the defibrinated plasma has been heated to 67° for 10 min. This may well be an inhibitory absorption of thrombin, but it is reversible and has no part in the antithrombin action of defibrinated plasma in the concentrations used in the present experiments, except when specifically mentioned. It may be described as an antithrombin effect, but it is not the one with which this paper is concerned. The subsequent progressive inactivation mentioned by this author is the one that has been studied here, and shown to be complex.

It is clear that any analysis of the kinetics of the reaction between thrombin and defibrinated plasma must await separation of the two factors involved. In the present situation, where the contribution of each is not known, mathematical formulation is not feasible.

SUMMARY

1. The antithrombin action of heparin on human thrombin has been studied, with and without the addition of defibrinated plasma.

2. Heparin in concentrations above 2·0 units/ml. forms a reversible complex with thrombin. This complex has less thrombin activity than free thrombin.

3. Heparin in concentrations above 0·1 unit/ml. catalyses a rapid combination between thrombin and a cofactor present in defibrinated plasma.

4. Heparin cofactor and the normal antithrombin of plasma are shown to be related but not identical, both occurring in the α-globulin fraction.

5. A theory suggesting that normal antithrombin consists of heparin cofactor as well as another independent thrombin inactivator is proposed to account for the observed facts.

My thanks are due to Dr R. A. Kekwick for much helpful advice and encouragement; to him and Miss M. Mackay for supply of human blood products; and to Dr I. A. Galloway, Ministry of Agriculture Research Station, Pirbright, for supply of ox blood.

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The Identification of Propionylcholine as a Constituent of Ox Spleen

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Propionylcholine has recently been identified by Banister, Whittaker & Wijesundera (1953) as a constituent of ox spleen. The identification was made on the basis of R_F value and physiological properties. It seemed desirable to confirm the identification by an unequivocal physical method such as infrared spectroscopy. Paper chromatography, the method of separation used in the earlier work, is not well suited for isolating propionylcholine in the quantities required, and an improved method of separation has been devised which makes use of chromatography on ion-exchange resins.

Ox spleen was originally selected by Banister *et al.* on account of the high content of acetylcholine or acetylcholine-like substances reported for it by Dale & Dudley (1929) and Chang & Gaddum (1933). It has not been possible to confirm these findings either in the earlier work or in the present study. This discrepancy and a possible explanation for it is considered in the discussion.

The work described in this paper was communicated to the XIXth International Physiological Congress, Montreal (Gardiner & Whittaker, 1953).

EXPERIMENTAL

Materials and methods

Ion-exchange resins. Amberlite XE-97 (lot no. 5258, 100–500 mesh) was washed by sedimentation in tap water to remove fines, transferred to columns and subjected to either of the following treatments:

(1) The resin was washed with N-HCl using double the volume required to give a metal-free effluent (i.e. flame test negative), then with water until the pH of the effluent exceeded 5 and finally washed with 0·1 M-NaH_2PO_4 until the pH of the effluent was the same as that of the influent.

(2) The resin was: (*a*) washed with N-NaOH, using double the volume required to change the tint of the resin from off-white to white; (*b*) removed from the column, washed by sedimentation with three to four changes of water and repacked in the column; then (*c*) washed with N-HCl, using double the volume required to change back the colour of the resin. (*d*) Step (*b*) was repeated. (*e*) Steps (*a*)–(*d*) were repeated twice. The column was then washed with water until the pH of the effluent was greater than 5 and equilibrated with 0·02 M-NaH_2PO_4.

Batches of prepared resin were stored under 0·1 or 0·02 M-NaH_2PO_4 until needed.

The basic resin Amberlite XE-98 (lot no. 4801) was powdered in a mortar, washed to remove fines, transferred to a column and converted into the perchlorate form by washing with 0·1 N-$HClO_4$ until free from chloride.

Amberlite XE-59 was converted into the hydroxide form by washing with 5% (w/v) NH_3 until free from chloride. Ammonia was removed by washing with boiled-out water until the pH fell below 7·4.

Other materials. Reagents and solvents whenever possible were A.R. A solution of $AgClO_4$ was prepared by the repeated evaporation *in vacuo* of $AgNO_3$ and $HClO_4$ at 100°, followed by neutralization of excess acid with Ag_2CO_3 and filtration. The methanol used in the final stages was Eastman Kodak 'Spectro' grade. Choline esters were as described by Banister *et al.* (1953).

Bio-assay of choline esters was carried out on the frog rectus abdominis muscle (Chang & Gaddum, 1933) or on the isolated guinea pig ileum (for details see Banister *et al.* 1953). When crude extracts were being assayed, allowance was made for interfering substances by the method of Feldberg & Hebb (1947), but when large numbers of assays had to be made on the fractions from a chromatographic run, this precaution was omitted, and the flanking procedure abbreviated somewhat.

The separate estimation of acetyl- and propionyl-choline in a mixture, particularly if other tissue constituents are present in appreciable quantities, presents some difficulty, especially as the relative potencies of the two esters in a given test system vary somewhat from one preparation to another and are influenced in an unpredictable manner by the composition of the extract. Nevertheless, it was necessary to have some method of measuring the activity of the various fractions in order to assess the efficiency of the purification procedure. The unit of activity adopted was the amount of synthetic acetylcholine perchlorate in m-μmoles giving an equivalent response on the frog rectus abdominis muscle (or guinea pig ileum, if specifically stated) when tested in a concentration range over which the dose/response curve of the preparation was approximately linear. Owing to the difficulties mentioned above, yields and recoveries based on activities measured in this way must be regarded as giving approximate indications only.

Chromatography. The columns were run at room temperature (25–35°). The effluent was collected in fractions of constant volume by means of an automatic fraction collector.

Chromatographic separation of choline esters

Choline and its esters, like other quaternary nitrogen bases, would be expected to exchange with the replaceable cations of acidic resins. Two procedures for the separation of these bases on acidic resins were considered: adsorption on the hydrogen form of the resin followed by displacement chro-

matography with acid solutions or adsorption on to the resin in its metal salt form (e.g. sodium form) followed by partition chromatography with solutions of salts of the metal. The first of these methods was found to be impracticable. Resins with sufficient salt-splitting capacity (i.e. sulphonic acid resins) caused rapid destruction of the esters, presumably because of the low internal pH of the resin particles. The second method permits the use of buffer solutions, but has the disadvantage that the active material has to be separated from a large excess of buffer salt. The strong acid resins again proved unsatisfactory, even with buffers close to the pH of optimum stability of the esters. Good separation (with recoveries of 90–100%) was however obtained with the carboxylic acid resin Amberlite IRC-50 or its fine-grain modification XE-97 and phosphate or acetate buffers at pH 4·3–4·5, values as near the pH of maximum stability of the esters (about pH 4) as is compatible with retention by the resin of a useful amount of base-binding capacity.

Experiments with synthetic esters showed that acetylcholine comes off the resin first, followed by propionylcholine, then by butyrylcholine. Succinylmonocholine and acetyl-β-methylcholine (β-acetoxypropyltrimethylammonium chloride) have about the same affinity for the resin as acetylcholine, but succinyldicholine is very firmly bound and needs solutions of pH 1–2 for its elution (unpublished work by I. A. Michaelson). Choline comes off slightly ahead of acetylcholine. When the pH of the eluting buffer is raised, the affinity of the resin for acetyl- and propionyl-choline first increases, then decreases, being maximal at about 5·5–6·0, and the degree of separation is diminished. The pH of maximum affinity for choline is somewhat higher (about 7·0) so that at this pH acetyl- and propionyl-choline emerge from the column together, followed by choline. Preliminary treatment with alkali, as in procedure (2), reduces the affinity of the esters for the resin, perhaps by removing material of low molecular weight, but gives sharper separation when used with a dilute (0·02 M) buffer. The alkali-treated resin was therefore preferred for runs with partially purified fractions. The separation obtained with synthetic acetyl- and propionyl-choline can be seen in Fig. 4.

Isolation of propionylcholine from ox spleen

Concentration of spleen bases. This was carried out according to the scheme outlined in Fig. 1. Up to the stage of reineckate precipitation, the procedure was essentially as described by Banister *et al.* (1953). Chromatography at this stage was unsatisfactory, apparently because columns of any practical size were overloaded by the large amount of inactive electrolytes present. These were partly eliminated by precipitating the tissue bases as their reineckates; further quantities were removed by decomposing the reineckates with silver perchlorate and making

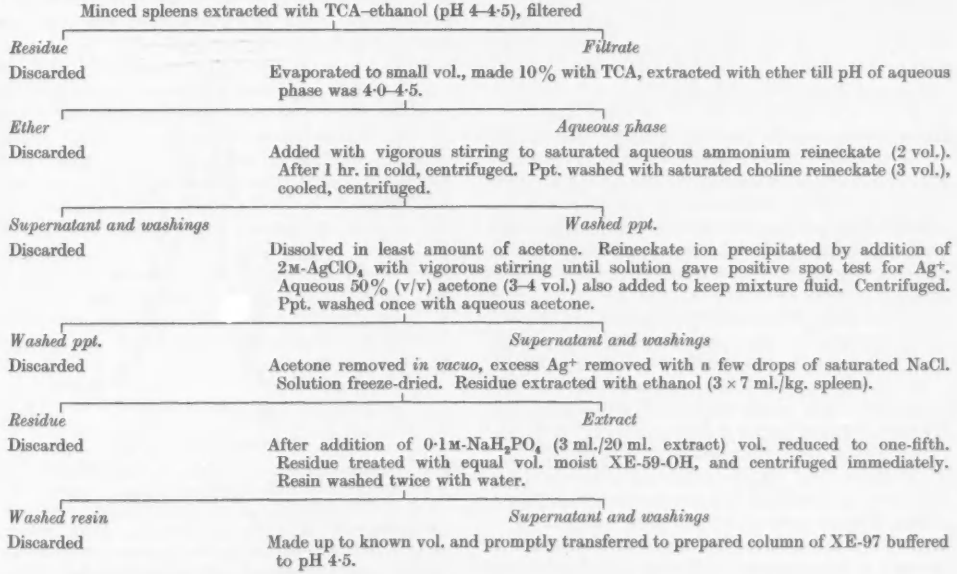
Minced spleens extracted with TCA–ethanol (pH 4–4·5), filtered
Residue
Filtrate
Discarded
Evaporated to small vol., made 10% with TCA, extracted with ether till pH of aqueous phase was 4·0–4·5.
Ether
Aqueous phase
Discarded
Added with vigorous stirring to saturated aqueous ammonium reineckate (2 vol.). After 1 hr. in cold, centrifuged. Ppt. washed with saturated choline reineckate (3 vol.), cooled, centrifuged.
Supernatant and washings
Washed ppt.
Discarded
Dissolved in least amount of acetone. Reineckate ion precipitated by addition of 2M-$AgClO_4$ with vigorous stirring until solution gave positive spot test for Ag^+. Aqueous 50% (v/v) acetone (3–4 vol.) also added to keep mixture fluid. Centrifuged. Ppt. washed once with aqueous acetone.
Washed ppt.
Supernatant and washings
Discarded
Acetone removed *in vacuo*, excess Ag^+ removed with a few drops of saturated NaCl. Solution freeze-dried. Residue extracted with ethanol (3 × 7 ml./kg. spleen).
Residue
Extract
Discarded
After addition of 0·1 M-NaH_2PO_4 (3 ml./20 ml. extract) vol. reduced to one-fifth. Residue treated with equal vol. moist XE-59-OH, and centrifuged immediately. Resin washed twice with water.
Washed resin
Supernatant and washings
Discarded
Made up to known vol. and promptly transferred to prepared column of XE-97 buffered to pH 4·5.

Fig. 1. Flow diagram showing the extraction of choline esters from ox spleen.

use of the sparing solubility of the inactive perchlorates (mainly creatine, potassium and choline) in ethanol.

A typical preparation was as follows. Six spleens (2·9 kg.) were removed from the animals 20–40 min. after death and immediately packed in chipped ice. Extraction was commenced in the laboratory cold room 1 hr. later. After removal of the capsule and adherent fat, the spleens were cut into strips and minced into ethanol (3 kg./kg. spleen). The cutting and mincing were carried out in the presence of chilled ethanol (cf. Dale & Dudley, 1929). After acidification with 50% (w/v) trichloroacetic acid (TCA), the mixture was left overnight in the cold room with stirring. After filtering through muslin and then through cellulose powder in a Büchner funnel, the filtrate was evaporated *in vacuo* at 37° and fat removed by extraction with ether. The aqueous residue (202 ml.) had a total activity of 4040 units. A considerable amount of activity was lost in the numerous assays at various stages, in the reineckate precipitation and the subsequent ethanol extractions (Fig. 1) leaving 1625 units (40%) for chromatography.

Chromatography of tissue choline esters. Fig. 2 shows the distribution of this active material in the effluent after chromatography on XE-97 prepared according to procedure (1). The initial peak of rectus-stimulating material was identified as choline on the basis of its position on the chromatogram, its predominantly rectus-stimulating activity (the choline:acetylcholine activity ratio for the frog rectus is 10 times that for the ileum), and its stability when boiled in alkaline solution. The next peak was identified as acetylcholine on the basis of its position on the chromatogram, the concordance of the rectus and ileum assays when synthetic acetylcholine was used as the standard, and alkali lability. The quantitative discrepancy between the rectus and ileum peaks is probably not significant, since some inactivation of the material may have taken place between the two series of assays, and there may have been substances present in some of the fractions which modified the response of the two organs to different extents. The third peak was identified as propionylcholine on the basis of its position immediately after acetylcholine on the chromatogram, its predominantly rectus-stimulating properties and its alkali lability. The activity recovered in the second and third peaks was 410 and 1040 units respectively, together comprising 91% of the alkali-labile activity before chromatography.

Histamine was identified as an ileum-stimulating substance whose alkali-stable activity was blocked by the antihistamine *N*-*p*-methoxybenzyl-*N'N'*-dimethyl-*N*-α-pyridylethylenediamine maleate (1 μg./ml.) (Merck and Co.) in fractions collected after 3·5–4·0 l. or in acid (0·1 N) eluates of columns from which choline esters had already emerged. Ninhydrin-positive material (basic amino acids) was present in fractions up to 200 ml. Butyrylcholine was not present in detectable amounts.

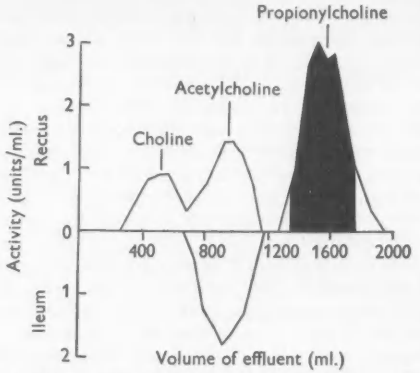

Fig. 2. Chromatography of concentrate of spleen tissue bases on XE-97. For definition of activity units see text. Column size, 27 × 2·4 cm.; eluting agent, 0·1 M-NaH_2PO_4; volume of fractions, 10 ml.; flow rate, 50 ml./hr.

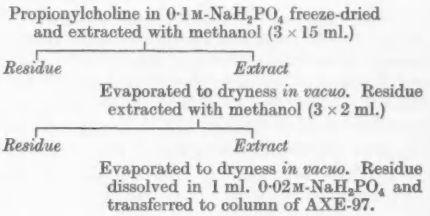
Propionylcholine in 0·1 M-NaH2PO4 freeze-dried and extracted with methanol (3 × 15 ml.)
Residue
Extract
Evaporated to dryness in vacuo. Residue extracted with methanol (3 × 2 ml.)
Residue
Extract
Evaporated to dryness in vacuo. Residue dissolved in 1 ml. 0·02 M-NaH2PO4 and transferred to column of AXE-97.

Fig. 3. Separation of propionylcholine from sodium dihydrogenphosphate.

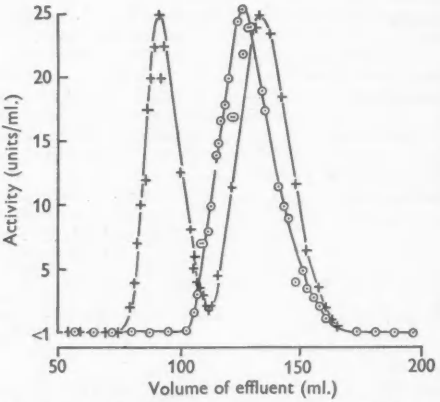

Fig. 4. Chromatography of spleen propionylcholine on AXE-97. Activity units as defined in text. Column size, 10·5 × 0·8 cm.; eluting agent, 0·02 M-NaH_2PO_4; volume of fractions, 1·2 ml.; flow rate, 5 ml./hr. Circles, spleen propionylcholine; crosses, calibration run with synthetic acetylcholine (0·4 μmoles) and propionylcholine (0·5 μmoles).

Fractions containing propionylcholine (blacked-in portion of Fig. 2, 910 units) were united with 2060 units of propionylcholine prepared in the same way from 5·4 kg. spleen. The combined material (2970 units in 1460 ml. 0·1 M-NaH_2PO_4) was divided into two portions, each of which was partially freed from sodium phosphate as shown in Fig. 3 and further purified by chromatography on AXE-97 using 0·02 M-NaH_2PO_4 as eluting agent. Fig. 4 (circles) shows the distribution of activity in the eluate from one of these runs. A calibration run (crosses) performed later with synthetic acetyl- and propionyl-choline shows that the active material from spleen runs like propionylcholine on the column. The slight rightward displacement of the control peak is accounted for by changes in rate of flow and ambient temperature. The total activity recovered after chromatography was 1650 units; this was 83 % of the activity placed on the columns, 56 % of the combined activity from the first chromatograms or 12 % of the ethanol-extractable activity of the original tissue.

Differential bio-assay of spleen propionylcholine. Although propionylcholine is relatively inactive on the ileum (2–10 % that of acetylcholine) it can be assayed on this preparation. Owing to the different relative potencies of different choline esters on the rectus and ileum (see Table 1, Banister *et al.* 1953) a comparison of the assay results obtained with the two types of muscle is one way of characterizing the esters (Chang & Gaddum, 1933). The assay results will, in general, only agree if the ester used as the standard is identical with the ester present in the 'unknown'. In Table 1 the results of two such differential assays are shown for the two samples of spleen propionylcholine prepared as described in the previous section. Using acetylcholine as standard, widely discordant results were obtained with the two assay systems, the 'unknown' appearing to have up to 60 times the activity when assayed with the rectus as with the ileum. When propionylcholine was used as a standard the two assays gave identical results.

Infrared spectrum of propionylcholine. Preliminary experiments with synthetic acetyl- and propionyl-choline perchlorates (kindly carried out by Dr A. R. H. Cole, Oxford University) had shown that the two esters could be distinguished on the basis of their infrared spectra. We had available 1650 units of spleen propionylcholine derived from a total of 8·3 kg. tissue and a further 210 units obtained in a pilot experiment from 1·7 kg. spleen; 1860 units in all, corresponding to about 300 μg. propionylcholine perchlorate. This precluded the use of a mulling technique and suggested the use of a spectrometer with reflecting microscope attachment (cf. Barer, Cole & Thompson, 1949). Dr Darwin L. Wood, of the University of Michigan, kindly undertook to place his microspectrometer at our disposal; his report is presented in the Appendix.

For spectroscopic examination it was necessary to separate the spleen propionylcholine from the buffer solution and mount it in a suitable manner. As spectroscopic identification of the naturally occurring ester isolated as the chloride by paper chromatography had been defeated, over 2 years ago, by the hygroscopic nature and instability of this salt, it was also considered essential to convert the ester into the more stable perchlorate. Accordingly, samples containing the ester (750 units in 50 ml.) were freeze-dried and the residue was extracted with methanol (2 × 10 ml.). After removal of the methanol, the residue was re-extracted with methanol (2 × 1 ml.); to this was added $CHCl_3$ (10 ml.). After 1 hr. in the cold, the mixture was centrifuged to remove sodium phosphate and the supernatant evaporated. The residue was transferred in a total volume of 5 ml. boiled-out water to an Amberlite XE-98-ClO_4 column (4 × 0·8 cm.) to convert the ester into the perchlorate. The effluent was evaporated and the ester transferred in a small volume of methanol to a 5 × 5 mm. silver chloride plate where it was deposited as a spot 2 mm. in diameter. The sample still contained a considerable amount of perchlorate derived from the NaH_2PO_4 of the eluting solution, but fortunately the inorganic ions present did not interfere with the identification of the biological material as propionylcholine.

Synthesis of propionylcholine by choline acetylase

The biological occurrence of propionylcholine raises the question of its synthesis. The most obvious route is via propionylcoenzyme A and the choline acetylase system, especially as enzymes are now known which convert the whole range of fatty acids, also succinic and benzoic acid, into acyl-coenzyme A derivatives. To test this point, the experiment summarized in Table 2 was performed. The source of choline acetylase was pigeon brain, already known as a rich source of cholinesterase (Whittaker, 1953). To identify the active component,

Table 1. *Differential bio-assay of spleen propionylcholine*

Sample	Choline ester used as standard	Bio-assay (m-μmoles/ml.) Rectus	Ileum	Ratio, rectus/ileum
I	Acetylcholine	2·75	0·1	27·5
	Propionylcholine	2·5	2·5	1·0
II	Acetylcholine	30	0·54	59
	Propionylcholine	30	32	1·07

Table 2. *Synthesis of choline esters by pigeon-brain choline acetylase*

An acetone powder of the brain was extracted with ice-cold 0·9% (w/v) NaCl for 1 hr. The supernatant after centrifuging (15 000 *g*) was treated for 0·5 hr. with 0·5 vol. XE-59-Cl in the cold (cf. Balfour & Hebb, 1952). The system contained in 2 ml., resin-treated extract from 40 mg. acetone powder, 50 μmoles cysteine, 100 μmoles KCl, 20 μmoles choline chloride, 1 μmole NaH_2PO_4, 25 μmoles NaF, 3·3 mg. adenosine triphosphate (Na_2 salt) and 60 μg. eserine (as sulphate); pH was adjusted to 7. Coenzyme A (Pabst Laboratories, Division of Pabst Brewery Co., Milwaukee, Wis.; 70% pure, 36 units) and Na acetate or propionate (20 μmoles) were added as indicated. The mixture was incubated for 1 hr. at 37°, brought to pH 4, boiled, centrifuged and the supernatant used for assays and chromatography. Activity expressed as μg. acetylcholine chloride/g. acetone powder.

	Activity after 1 hr.	Chromatographic identification of active material
Extract + CoA	145	Acetylcholine
Extract + acetate	225	Acetylcholine
Extract + CoA + acetate	3100	Acetylcholine
Extract + CoA + propionate	1100	Propionylcholine + trace of acetylcholine

extracts were freed from choline by chromatography on AXE-97 at pH 7·4; the active fractions were freeze-dried and run on AXE-97 at pH 4·5 to separate acetyl- and propionyl-choline. As seen in Table 2, propionate gave rise to propionylcholine in this system; the trace of acetylcholine probably arose from acetyl donors which, as shown by the first result recorded, were present in the preparation. Berman, Wilson & Nachmansohn (1953) have recently reported that purified squid-ganglion choline acetylase can synthesize propionylcholine from propionylcoenzyme A or an appropriate donor system; their product was not isolated, however.

DISCUSSION

Propionylcholine from ox spleen has now been identified as such by chromatography on paper and on columns of ion-exchange resin, by differential bio-assay and by infrared spectroscopy. The technique of chromatography on ion exchange resin has several advantages over paper chromatography (Whittaker & Wijesundera, 1952) for separating choline esters; the separation is sharper and less affected by impurities, and the recoveries are excellent. The chief disadvantages for our purposes were the difficulty of separating the esters from the buffer solution and the dilution of the extract which occurs during elution. The first of these was not entirely overcome, and the sample used for infrared spectroscopy contained a large amount of inorganic perchlorate derived from buffer salt. Fortunately the perchlorate ion did not absorb in the critical portion of the spectrum and so did not interfere with the identification. In the purification procedure, high yields were regarded as secondary to the attainment of a homogeneous product (thus, for example, only the central portion of the propionylcholine peak in Fig. 2 was worked up); nevertheless, the amount of propionylcholine isolated (300 μg. from 10 kg. tissue corresponding to over 11% of the ethanol-extractable activity of the original spleen) does not seem unreasonable in view of the losses incurred in the reineckate precipitation and in the numerous assays, extractions and transfers. The yield of propionylcholine cannot be evaluated exactly owing to the difficulty of estimating the amount present in the original tissue, but Fig. 2 shows that about 60% of the total activity at this stage was due to propionylcholine and 30% to acetylcholine. Since propionylcholine is 1·5–2·0 times as active on the frog rectus as acetylcholine, this suggests that the extract contained at least as much propionylcholine as acetylcholine. If it is assumed that losses before chromatography affected both esters equally, the overall yield of propionylcholine might be as high as 20–25%.

Our estimate for the propionylcholine content of ox spleen is somewhat higher than that given by Banister *et al.* (1953). However, Banister (unpublished) has recently confirmed that part of the propionylcholine in spleen extracts may travel with acetylcholine on paper chromatograms, and the impression was also formed in the earlier work that propionylcholine (as the chloride) was less stable on paper chromatograms than was acetylcholine. Both these factors, which would lead to underestimation of the proportion of propionylcholine, are absent from the present work.

'F component' (Banister *et al.* 1953) was not detected in the effluents from the columns even after prolonged chromatography. However, some evidence was obtained that it was strongly adsorbed on the resin and could, like succinyldicholine and histamine, be eluted with acid. Work on the separation and identification of this substance is continuing.

The amount of 'acetylcholine' (i.e. total acetylcholine-like activity as assayed by the frog rectus) extracted from ox spleen by acid-ethanol in the experiments described in this paper was 1·6 units/g. tissue, equivalent to 0·3 μg. acetylcholine chloride/g. This is the same order of magnitude as has been observed before (0·3–0·9 μg./g.), but is much lower than that reported for horse and ox spleen by Chang & Gaddum (1933) (4–30 μg./g.), using the

trichloroacetic acid technique. Ambache (personal communication) has also obtained results lower than those reported by Chang & Gaddum. Differences in extraction procedure do not account for the discrepancy, as our values using trichloroacetic acid as the extractant were not more than 30 % higher than those obtained with acid-ethanol. Dale & Dudley (1929), using the acid-ethanol method, also reported high 'acetylcholine' levels in ox spleen, though their paper is somewhat vague as to the range of values actually found. Their range for horse spleen was 5–30 μg./g. (p. 101 of their paper); one batch of 32 kg. had 10·4 μg./g. (p. 111) and they state (p. 108), 'there is no reason for supposing that the yield...(of acetylcholine from ox spleen) would have been less under similar conditions'. However, it is possible to infer from an earlier passage (p. 99) that the acetylcholine content of a 50 kg. batch of ox spleen was only 2 μg./g. Their figures for horse spleen were confirmed by the isolation of acetylcholine from this tissue in relatively large quantities, but no such confirmation exists for ox spleen.

In order to exclude inadequacies in extraction technique as an explanation for the discrepancy between our own results and those of the Dale school, we have recently examined two horse spleens. Using the trichloroacetic acid method of extraction the 'acetylcholine' content was 6·8 and 7·6 μg./g. respectively; the acid-ethanol procedure gave results about 20 % lower (cf. Chang & Gaddum, 1933). These results are within the range given by Chang & Gaddum and are fairly close to the value reported by Dale & Dudley for their 32 kg. batch of spleen tissue. On submitting the active material to chromatography, almost 100 % of the activity behaved like acetylcholine on the column and in the subsequent differential assays. No other ester of choline was present in detectable amounts.

Aside from possible changes in diet, breeds and methods of slaughtering during the past 20 years, we believe that failure to allow for potentiating substances present in the crude tissue extracts may account, in part, for the high values of the earlier workers. We have found that, when no allowance is made for alkali-stable potentiating substances by the Feldberg technique, values nearly 3 times higher than the 'true' ones may be obtained. This would bring the upper part of our range near the lowest value given by Chang & Gaddum. By contrast, the amount of potentiation in the horse-spleen extracts was much less (16 and 0 % respectively). Now, neither Chang & Gaddum nor Dale & Dudley allowed for potentiating substances and in the case of the rabbit jejunum assays performed by the latter, one such substance, histamine, was admittedly present (they describe its isolation from ox spleen). We therefore believe our values represent the true ones obtainable under present-day conditions with currently acceptable assay techniques.

SUMMARY

1. The separation of propionylcholine from ox spleen by an improved method involving chromatography on ion-exchange resins is described.

2. The spleen propionylcholine was identified as such by its chromatographic and physiological properties and by its infrared absorption spectrum.

3. Reasons are advanced for believing that the values obtained for the total concentration of acetylcholine and acetylcholine-like substances in spleen tissue, though lower than those reported by earlier workers, represent the true values obtainable with modern assay techniques.

This investigation was supported by a research grant (H-1254) from the National Heart Institute of the National Institutes of Health, U.S. Public Health Service and by Hoffmann-La Roche Inc. We wish to express our gratitude to the following: the Rohm and Haas Company for gifts of resins, Herman Kemper & Sons for generous supplies of ox spleen, Mr D. Dalessandro for horse spleen, the May Institute of Medical Research for the use of their freeze-drying apparatus, the U.S. Public Health Service and Hoffman-La Roche, Inc. for financial support, Miss R. J. Banister and Mr I. A. Michaelson for help with some of the experiments.

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APPENDIX

The Identification of Spleen Propionylcholine by Infrared Microspectroscopy

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As a preliminary to the study of the sample of spleen propionylcholine, the spectra of synthetic acetylcholine and propionylcholine perchlorates were recorded, together with those of two possible impurities in the biological material, choline perchlorate and sodium perchlorate. The spectra of the pure compounds were measured with a Perkin-Elmer Model 21 double-beam infrared recording spectrophotometer, using standard mulling techniques for all but the choline perchlorate sample. The mulling agents were Nujol (paraffin oil) and hexachlorobutadiene; since one agent is clear where the other absorbs, the sample bands obscured by one agent could be observed in the mull with the other. The sample of crystalline choline perchlorate was observed to be unusually malleable, and was rolled out into thin self-supporting films, or alternatively, rolled on optical silver chloride sheets to the thickness required for spectroscopic samples.

The spectra of the four compounds recorded in this way are shown in Fig. 1. The curves have been redrawn so that the bands of the mulling agents do not appear. It will be noted that there are differences in the spectra of the two esters which are remarkable in view of the slight difference in their structure. In particular the differences in the region from 7·5 to 12 μ. should be noted, since these are the bands which were used in making the identification of the spleen-extract ester sample. Below the spectra of the esters are shown the spectra of choline perchlorate and sodium perchlorate. It will be noted that the strong band of the free hydroxyl group of choline which appears at 2·87 μ. is absent from the spectra of the ester sample shown above, indicating that choline is not an impurity in the synthetic samples. Since hydrated sodium perchlorate (bottom curve in Fig. 1) has an OH frequency due to the water of hydration lying very close to that of the OH frequency of choline (2·82 and 2·87 μ. respectively), it is not possible to detect the presence of choline in ester samples containing sodium perchlorate as an impurity, using this band as a criterion. There are other bands, however, by which choline can be detected in the presence of sodium perchlorate, and although these are much weaker and the criterion correspondingly less critical, no evidence for the presence of choline in the spleen-extract sample was found.

The spectrum of sodium perchlorate shown at the bottom of Fig. 1 indicates that the broad band near 9 μ. common to all the perchlorate spectra is due to the perchlorate ion. This band does not obscure the most important differences between the spectra of the two esters in the top two curves. It was concluded that neither choline nor sodium perchlorate would interfere in the spectroscopic identification of propionyl- or acetyl-choline perchlorates. To establish that the identification could be made in the presence of a considerable excess of sodium perchlorate, two pairs of mixtures of the two esters and sodium perchlorate were prepared by grinding in a mortar. The spectra of these samples run as mulls in the double-beam spectrometer showed that a mixture containing 4 % of ester was too dilute to discriminate between the acetyl and propionyl esters. In 18 % samples, however, it was quite easy to tell which was which and the differences in the spectra agreed fairly well with those recorded from mulls of the pure compounds, although the agreement was not complete, indicating compound formation between the ester and the sodium perchlorate.

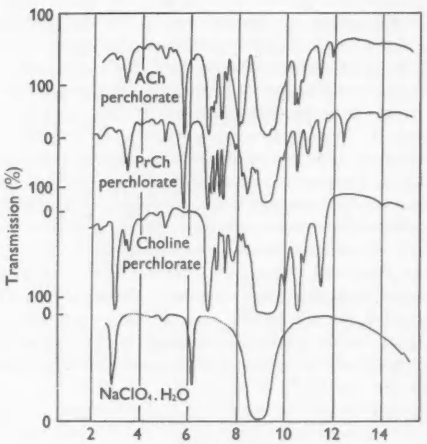

Fig. 1. Infrared spectra of acetylcholine (ACh), propionylcholine (PrCh) and choline (all as perchlorates) and of sodium perchlorate monohydrate.

With this established, the spectra of the mixtures of ester and sodium perchlorate were recorded again using an infrared microspectrometer previously described (Wood, 1950). The microspectra show somewhat less resolving power than the double beam spectra and the region showing the spectroscopic differences between the esters is given in Fig. 2. The other regions of the spectrum where differences between the two esters also occurred are not included, because for various reasons the microspectra were considered unreliable (e.g. water vapour in the 6–7·5 μ. region). In comparing the two upper curves in Fig. 2 it will be noted that the acetyl ester has the band at 8·05 μ. lacking in the propionyl ester and the propionyl ester has strong absorption at 8·35 μ. where the acetyl ester has a region of maximum transmission. On the other side of the broad 9 μ. band due to perchlorate the acetyl ester has a broad multiple band near 10·5 μ. while the propionyl ester has a single sharp peak at 10·50 μ.

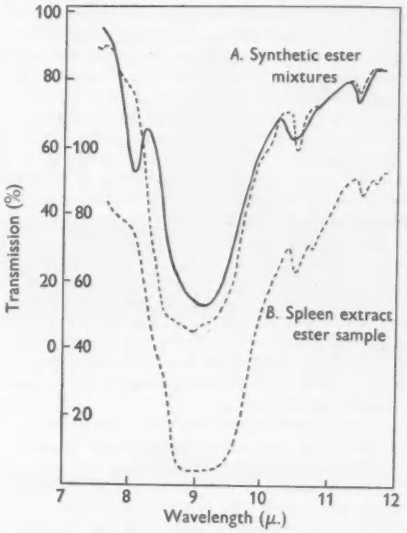

Fig. 2. Microspectra of *A*, synthetic acetyl- and propionylcholine perchlorates as mixtures with sodium perchlorate (19 and 16·5% ester respectively) and *B*, spleen ester sample. ——, acetylcholine perchlorate mixture; - - - - -, propionyl perchlorate mixture.

The curve *B* in Fig. 2 is the microspectrum of the sample of the biological extract thought to be propionylcholine perchlorate, and containing approximately 100 μg. of active material. This curve lacks the acetyl ester band at 8·04 μ., but has the propionyl ester band at 8·35 μ. (as a shoulder). In addition, on the other side of the intense perchlorate band at 9 μ. the natural ester curve has a single peak at 10·50 μ. similar to that of the synthetic propionyl ester, and in contrast to the broad multiple band of the acetyl ester in the same region. With the exception of the small peak at 10·75 μ. in the natural extract the spectrum of the latter is identical with that of the synthetic propionylcholine perchlorate. The 10·75 μ. band is attributed to an unidentified impurity from the extraction procedure. On the basis of the infrared spectrum, the conclusion is that the extracted ester is indeed propionylcholine.

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The Application of Four Methods for Assessing Protein Homogeneity to Crystalline β-Lactoglobulin: an Anomaly in Phase Rule Solubility Tests

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(*Received* 18 *November* 1953)

In order to follow the purification of a protein a reliable and sensitive method for determining the number and amount of components present in different samples must be available. Ultracentrifugal, electrophoretic, immunological and solubility tests were carried out with crystalline β-lactoglobulin in order to find such a method for following further purification of this protein. The results of these tests are presented in the first part of this paper and the usefulness of each is considered.

In the second part of the paper the occurrence under certain conditions of an anomaly in the 'variable solvent' solubility test for protein homogeneity (Falconer & Taylor, 1946; Roche & Derrien, 1947; and Derrien, 1952) is described and discussed. This appears to be the first time that such an

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anomaly in phase rule solubility tests has been demonstrated experimentally, although the theoretical possibility of a related phenomenon in 'constant solvent' solubility tests has been described (Northrop, Kunitz & Herriot, 1948).

EXPERIMENTAL METHODS

Protein. Two samples of crystalline β-lactoglobulin were used: LG I, a sample prepared by a modification of Cecil & Ogston's (1949) method (Smithies, 1953) and recrystallized 9 times from aqueous NaCl solutions by dialysis: and LG II, a sample prepared by uniting approximately equal quantities of once recrystallized lactoglobulin, prepared by the above method, with a 3 times recrystallized sample prepared by the method of Cecil & Ogston (1949) and recrystallizing the mixture once more. No difference between the two samples could be established in tests in which both were used. The crystals were stored at 4° under distilled water saturated with toluene.

Physical methods. In the ultracentrifugal test a Spinco ultracentrifuge was used, by the courtesy of H. F. Deutsch, Department of Physiological Chemistry, Wisconsin University. The electrophoretic studies were made with the apparatus and technique in use by R. A. Alberty, J. W. Williams and their co-workers at Wisconsin University (see, for example, Alberty, Anderson & Williams, 1948; and Baldwin, Laughton & Alberty, 1951).

Immunological methods. In the immunological work two samples (one completely free from visible haemolysis products) of a pooled serum were obtained from two rabbits, following seven and nine subcutaneous injections (spread over 3 weeks) each of 1 ml. of an approximately 1 g./100 ml. solution of LG I in 0·15 M-NaCl. The rabbits were bled from the ear 5 days after the last injection. The serum, after the addition of merthiolate to 1 part in 10000, was stored at −20° until required.

The precipitation curve for LG I against these antiserum samples was determined as follows. A series of tubes was set up each containing 0·2 ml. of the serum and 0·2 ml. of LG I solution in approximately isotonic pH 7·4 phosphate–NaCl buffer. The lactoglobulin solutions covered dilutions of from 1 part in 50 (w/v) to 1 in 3280000. After mixing and standing at room temperature for 2·5 hr. the relative amount of precipitate was determined for the contents of each tube by measuring the absorption of light of wavelength 346 mμ. in a Beckman spectrophotometer, using a suitably masked Perspex cell of 0·3 ml. capacity. In the region of maximum precipitation the supernatant solutions (separated by centrifuging after having stood for 24 hr. with the precipitates) were tested for excess antigen and antibody by means of ring tests with the undiluted serum and 1 in 50000 LG I respectively.

Solubility test methods. In the solubility work the 'variable solvent' solubility test was used, and the salt precipitation curves were determined as follows, with unimportant differences in the earlier tests. The protein sample to be tested was dissolved in 2·4 M-NaCl and the solution centrifuged at 20000 *g* to ensure its clarity. Equal volumes of the protein solution and centrifuged 2·4 M sodium acetate buffer of the required pH were mixed, and 0·2 ml. portions of the mixture transferred to dry glass test tubes (10 × 75 mm.) previously cleaned with alkali, hot chromic acid and distilled water. To each tube 1 ml. of $(NH_4)_2SO_4$ solution was then added, the concentration of $(NH_4)_2SO_4$ being increased stepwise from tube to tube. The acetate concentration in the resulting solutions was 0·2 M so that they were adequately buffered. The tubes were sealed with Parafilm sheet (Marathon Corporation, Menasha, Wisconsin, U.S.A.), as corks give impurities absorbing in the ultraviolet; their contents were mixed by inversion. The tubes were transferred to a constant temperature bath at 25° until ready for the solubility determinations, when the contents were once again mixed by inversion and centrifuged for 20 min. at about 20000 *g* in a Servall SS 1*a* centrifuge. (Filtration was avoided as all types of paper tried contained water-soluble substances absorbing in the ultraviolet). From 50 to 67 % of the supernatant of each tube was removed without disturbing the solid. In doing this the tube was held at approximately the same angle and position as during centrifuging and the supernatant was withdrawn by means of an acid cleaned dry Pasteur pipette with the tip of the pipette almost touching the lowest part of the tube opposite the pellet. The optical densities of the supernatant solutions were determined at 278 mμ. in a Beckman spectrophotometer fitted with a mask limiting the light beam to a size of 2 × 6 mm. so that a modified quartz cell of solution size 3 × 10 × 15 mm. could be used. The initial protein concentration was adjusted so that the maximum optical densities of these final solutions were between 0·40 and 0·45. This corresponds to a concentration of about 0·05 g. lactoglobulin/100 ml., so a test of twenty-four tubes could be made with less than 20 mg. of protein.

The precipitation curves were found to be sensitive to small changes in the system, for example in the buffer composition, consequently in comparing any two curves only one component at a time (protein, buffer or $(NH_4)_2SO_4$ solutions) was varied. The procedure described facilitates this. The initial protein concentration was also kept the same in any two tests to be compared, as it was found that the precipitation zone of a protein is influenced by the total amount of protein present (cf. Falconer & Taylor, 1947). When checking a result all solutions were freshly made up to minimize accidental effects.

The precision of the test was later found to be improved and the labour of setting up greatly reduced by the use of automatic pipettes, but the results here presented were obtained with simple calibrated glass pipettes.

RESULTS AND DISCUSSION

(1) *Homogeneity tests*

Fig. 1 shows a tracing of a photograph of the boundary formed after 2·5 hr. of centrifuging at 59780 rev./min. of an approximately 1 g./100 ml. solution of LG I in 0·2 M sodium acetate buffer, pH 5·1, at an average temperature of 24·1°.

Fig. 2 illustrates the electrophoretic patterns obtained with various solutions of LG II at pH values close to the isoelectric point of the protein.

Fig. 3 shows the results obtained by the author in two determinations of the precipitin curve for LG I against two samples of its rabbit antiserum. The relative amount of precipitate formed is plotted against the final LG I concentration present. The broken line and half filled points show some com-

parable results obtained by Deutsch (1950) with a preparation of crystalline lactoglobulin made by the method of Cecil & Ogston (1949). The results of tests for excess antigen and antibody are also shown. The dotted line and open points show Deutsch's results with material prepared by the method of Palmer (1934).

Fig. 4 shows the 'variable solvent' solubility (salt precipitation) curve obtained at pH 4·8 with LG II. It is the combined results of two determina-

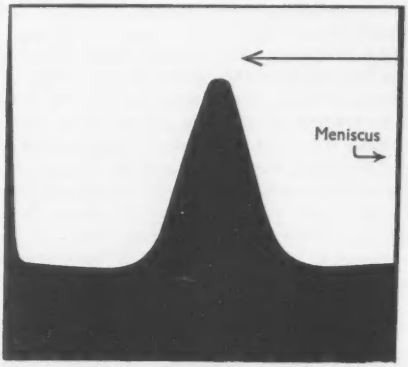

Fig.1. Sedimentation diagram of an approximately 1 g./100 ml. solution of LG I in 0·2 M sodium acetate buffer, pH 5·1. Time 2·5 hr. Speed 59 780 rev./min. Temperature 24·1°.

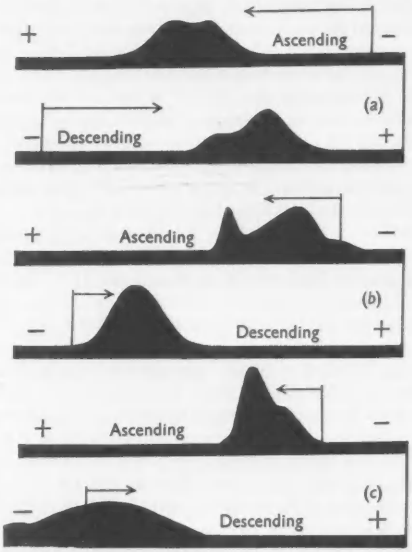

Fig. 2. Electrophoretic patterns of LG II in various solutions. The initial boundary positions are indicated by vertical lines and the directions of migration by arrows. (*a*) Approximately 1% (w/v) protein; 0·05 M-NaCl, pH 5·60±0·05, or 0·05 M Na acetate, pH 5·50±0·05. (*b*) 0·7 or 1·3% protein; 0·1 M-NaCl, pH 5·50±0·05. (*c*) 1·3% protein, 0·09 M-NaCl+0·01 M Na acetate, pH 5·15±0·05.

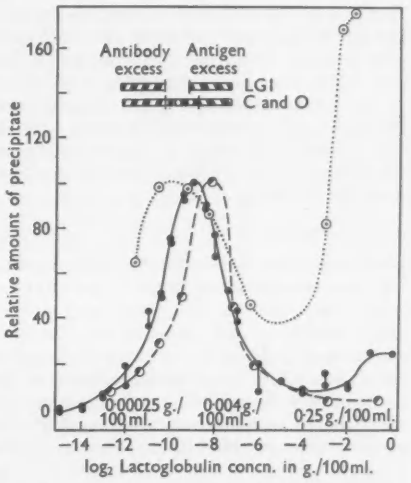

Fig. 3. Precipitation curves for various crystalline lactoglobulin samples against their corresponding antisera. The author's results with LG I are shown by a solid line and filled points. Data obtained by Deutsch (1950) are shown by a broken line and half filled points (for lactoglobulin prepared by the method of Cecil & Ogston, 1949), and by a dotted line and open points (for a sample prepared by the method of Palmer, 1934). The results of tests for excess of antibody and antigen on the supernatants for the LG I and Cecil & Ogston lactoglobulin (C and O) experiments are shown above the curves. The relative amount of precipitate is calculated as 100 × increase in optical density at 346 mμ. over blank ÷ maximum increase. The final LG I concn. present is expressed as $\log_2$ LG I in g./100 ml. The vertical axis scale for the Deutsch data is 100 × mg. N in precipitate ÷ mg. N in precipitate at first maximum.

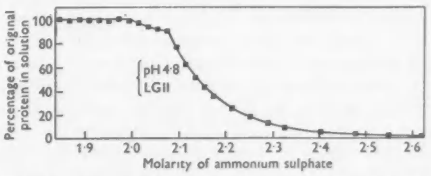

Fig. 4. The 'variable solvent' solubility (salt precipitation) curve for LG II at pH 4·8. The original solutions contained approximately 0·05 g./100 ml. of protein and were 0·2 M in sodium acetate, with sufficient acetic acid present to give the required pH. The curve gives the combined results of two experiments with equilibrium times of 2 days.

tions with equilibrium times of 2 days. The vertical axis represents the percentage of the original protein remaining in solution. This figure is obtained from the optical densities of the supernatant solutions at 278 mμ. (corrected for the blank with no protein and expressed as the percentage of the optical density for the tubes in which no precipitation has occurred) on the assumption that the extinction coefficients of each of the protein components is the same. Since Tilley (private communication) finds no significant change in the ultraviolet absorption of lactoglobulin on fractionation, this assumption is probably justified. The horizontal axis shows the corresponding ammonium sulphate concentration in the supernatant solutions.

Purity of lactoglobulin

Inspection of the ultracentrifugal result presented in Fig. 1 shows no evidence for the existence of more than one component in LG I. Confirmation of this result is provided by Baldwin (personal communication), who was unable to find evidence for more than one component from detailed mathematical analysis of the boundary shape in the ultracentrifuge at 28° of solutions of recrystallized β-lactoglobulin also prepared by the modified Cecil & Ogston method, although he points out that even with this analysis two components present in equal amounts could only be detected reliably in this case if their sedimentation coefficients differed by $>10\%$.

Since in the ultracentrifuge at these temperatures crystalline β-lactoglobulin appears already homogeneous, within the present resolving power of the method, use of the ultracentrifuge to follow further purification would be pointless.

The electrophoretic studies indicate the presence of at least two components in LG II. These lactoglobulin solutions, however, showed marked anomalies in electrophoresis, as can be seen from the illustrative results presented in Fig. 2. The ascending and descending patterns differ widely, and small changes in the system bring about large changes in the apparent component analysis. Thus an unequivocal analysis of the component composition of various lactoglobulin samples by electrophoresis would clearly require extensive investigations for each sample at a series of protein concentrations and in various buffers. It would thus be impractical to use the method to follow further purification of lactoglobulin. This conclusion is supported by more extensive electrophoretic studies made by Tilley (private communication) with the same protein.

Of the various immunological methods available for investigating protein homogeneity the determination of the precipitin curve for the protein against its rabbit antiserum was chosen for this study because of its quantitative nature. An immunologically pure protein should give a symmetrical precipitin curve with a single maximum when the amount of precipitate formed is plotted against the logarithm of the amount of protein antigen added to a constant amount of its rabbit antiserum. An equivalence zone should be observed over which no excess of either antibody or antigen is detectable in the supernatant solution after removal of the precipitate.

Of the precipitation curves plotted in Fig. 3, that due to Deutsch with the Palmer lactoglobulin shows a clear indication of the existence in this preparation of more than one component, in that more than one maximum is present. The author's results with LG I and Deutsch's with the Cecil & Ogston lactoglobulin are closely similar and, as only one maximum is clearly seen, offer little evidence for the presence of more than one component in these preparations. Consequently, this immunological test was also regarded as unsatisfactory for following further purification of lactoglobulin.

It should be pointed out however that, while Deutsch in his immunological test on the Cecil & Ogston lactoglobulin obtained no equivalence zone, in the present work with LG I an equivalence zone was observed (see Fig. 3). The significance of this observation as a criterion of the greater immunological homogeneity of LG I is, however, rendered uncertain for two reasons: (*a*) there is a small rise in the precipitin curve for LG I, which should not occur with an immunologically pure protein, at very high LG I concentrations (250 times that at the maximum) in the one experiment carried beyond 0·25 g. LG I/100 ml.; and (*b*) that as the demonstration of an equivalence zone is the result of negative tests for antigen and antibody excess it may only reflect insufficiently sensitive tests for their presence.

Previous workers (see, for example, McMeekin, Polis, Dellamonica & Custer, 1948, who give earlier references) have found evidence for the inhomogeneity of crystalline β-lactoglobulin from solubility studies, using the 'constant solvent' method. Their results do not, however, permit a quantitative estimation of the components present. For the solubility studies here described the 'variable solvent' solubility test was therefore chosen as it can more readily provide this quantitative data.

The 'variable solvent' solubility curve for LG II plotted in Fig. 4 was obtained at pH 4·8. Similar curves were also obtained with LG II at pH's 4·5 and 5·1, and with LG I at pH's 4·8 and 5·1. This type of curve, in which two breaks are clearly visible, could regularly be obtained with lactoglobulin at any of these pH values with equilibrium times ranging from 1 to 9 days. Examination of the precipitates obtained showed them to consist in part of completely amorphous (gelatinous) material, and in part of solid, the structure of which, even when best defined, could not be fully resolved by an

oil immersion objective, but which had the appearance of short branched strings of beads. The gelatinous precipitate appeared first in the course of the precipitation and may have corresponded to the material represented by the initial break in the curves.

In view of the way in which these curves could repeatedly be obtained with either lactoglobulin sample at three different pH's and with considerably different equilibrium times it is concluded that the two breaks represent two 'true' components present in lactoglobulin as prepared by the Cecil & Ogston method or its modification.

The precipitation curves follow the form described by Cohn (1925), viz.

$$\text{Solubility} = e^{(\beta - \alpha . c)}$$

(where α and β are constants, and c is the salt concentration). The smooth curves in Fig. 4 were drawn to fit this relationship.

Application of variable solvent solubility test

In view of the relative simplicity and reliability of this 'variable solvent' solubility test it was regarded as the most suitable method of the four tested for following attempts at fractionating lactoglobulin. Two applications of the method may be quoted. The first of these was to find out whether the repeated recrystallizations during the preparation of LG I had affected its component analysis. A sample of crystalline lactoglobulin was therefore prepared by the modified Cecil & Ogston method but was not recrystallized. The solubility curve at pH 5·1 of this material was compared with that of LGI, the 9 times recrystallized sample. No difference between the two samples could be demonstrated in this solubility test, despite the fact that LG I had been stored for nearly a year and the once-crystallized material for less than 2 weeks. Some data presented later in this paper throw light on the ineffectiveness of these repeated recrystallizations in modifying the component analysis of the protein.

The second application of the method was to follow an attempt at fractionating β-lactoglobulin by using the change with temperature of the solubility of the protein in dilute salt solutions. Several saturated solutions of LG I in 0·05 M-NaCl were prepared at 25°, and centrifuged to ensure their clarity. An equal volume of 0·05 M-NaCl was added to each and the mixture cooled to 0°, when gelation occurred in a few minutes. After standing at 0° for varying times from 2 to 5 hr., the gels (usually about 16 % of the total material) were separated as precipitates in a cooled centrifuge. In all but one out of the five gelation experiments of this type which were made, no significant difference between the precipitate and supernatant could be demonstrated by the solubility test. To illustrate these experiments, the solubility curves determined at pH 5·1 with the products of a typical gelation (IV), and of the one (II) in which appreciable fractionation was obtained, are presented in Fig. 5. The atypical result could not be repeated but serves to show that fractionation can be followed by this test when it occurs.

It will be noted that the curves shown in Fig. 5 suggest the presence of three components in β-lactoglobulin. The one present in a small amount was not observed in the earlier solubility experiments quoted (see, for example, Fig. 4) because at that time random errors, probably in the pipetting, obscured its presence. Insufficient experiments were performed however to exclude the possibility that this component was a small amount of denatured material.

These solubility tests suggest the presence of two (possibly three) components in crystalline β-lactoglobulin prepared by the modified Cecil & Ogston method. The observed proportion of the components is approximately 1 : 5 : 20, assuming that each has the same extinction coefficient (see above). These proportions will, however, only represent those present in the original solution provided that the precipitation of any one of the components does not cause the

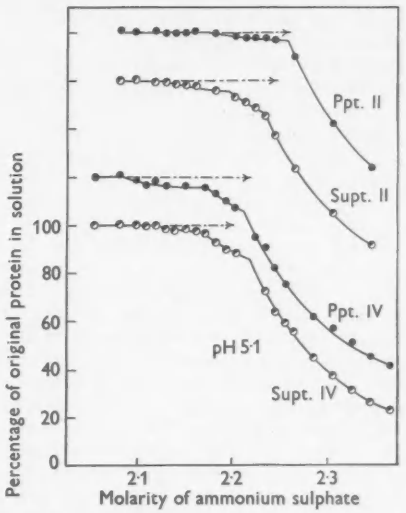

Fig. 5. The solubility curves at pH 5·1 for the supernatant solutions and precipitates from two gelation experiments with LG I. The upper two curves are the combined results of three determinations of the solubility curves for the products of gelation experiment no. II, with equilibrium times of 5–8 days; the lower two are the results of a single determination of the curves for the products of gelation IV, with an equilibrium time of 3 days. The vertical axis is numbered only for supernatant IV to avoid confusion.

co-precipitation of any of the others. This condition is probably fulfilled by the present system at pH 5·1, as equivalent results can be obtained at two other pH values.

(2) *An anomaly in the 'variable solvent' solubility test for protein homogeneity*

Fig. 6 shows the results obtained with LG II at pH 5·4 in the 'variable solvent' solubility test in a single determination of the solubility curve with an equilibrium time of 2 days. Fig. 7 shows two repetitions of this experiment with LG I. In the first of these, five sets of tubes were set up and the solubilities were measured at daily intervals from 1 to 5 days. In the second repetition, two sets of tubes were used and the solubilities were measured after 2 and 5 days.

Fig. 8 shows some of the results obtained with LG I at pH 5·7. Determinations of the solubilities were made 1, 2, 3, 6, 9, 13, 20 and 30 days after setting up, with several duplicates, but for clarity of presentation only the results after 1, 3, 13 and 30 days are plotted.

In part (1) of the Results it was established that lactoglobulin shows two main components in the 'variable solvent' solubility test (see Fig. 4) at pH values of 4·5, 4·8 and 5·1, and that the type of curve obtained was unaffected by the time allowed for equilibrium to be established.

At pH 5·4, however, with both LG I and LG II, a discontinuity suggesting the presence of a considerable amount of an additional 'component' is seen (the regions of open points in Figs. 6 and 7), and in Fig. 7 it can be seen that in this region the two repetitions of the experiment diverge considerably. This is in marked contrast to the other regions of the curve where good agreement between the two repetitions was found. Furthermore, the precipitates in the tubes indicated by the open squares in Fig. 6 were noticeably different in character (appearing granular) from those in the other regions of the curve; and examination under the microscope of precipitates from duplicate tubes showed them to contain clusters of parallel needle-like crystals. This observation was regarded as evidence that the additional break in the curves at pH 5·4 is due to the presence of a different (crystalline) form of the precipitate, stable at this pH only over a narrow range of salt concentrations, and does not represent a 'true' additional component. The variability of the results in this region supports this conclusion. It can be explained as due to differences in the proportion of the total material in the crystalline form, which proportion is probably very sensitive to small differences in the system such as may be inadvertently made in repeating an experiment.

Yet more confirmation of the correctness of these ideas is found in the results obtained at pH 5·7, at which pH the crystalline form is the stable solid phase at all salt concentrations, though at short equilibrium times the amorphous type of curve can be seen. The results of an extensive experiment at pH 5·7 with LG I are shown in Fig. 8. Observations of the form of the precipitate were made on every tube and the approach to final equilibrium carefully followed.

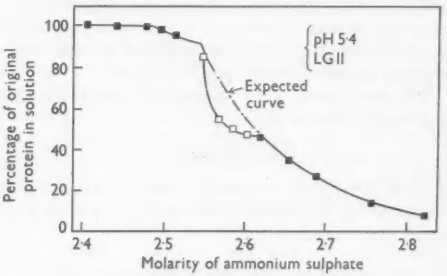

Fig. 6. The solubility curve for LG II at pH 5·4. A single determination with an equilibrium time of 2 days.

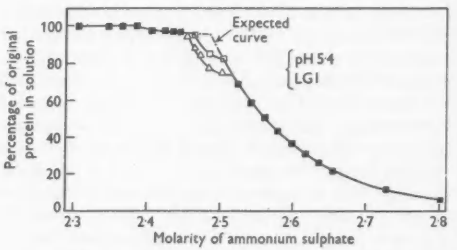

Fig. 7. Solubility curves for LG I at pH 5·4. △ are the results of an experiment consisting of five determinations with equilibrium times of 1–5 days; □ are the results of a repetition of the experiment with two determinations with equilibrium times of 2 and 5 days. ■ the combined results of both experiments where no difference between them was apparent.

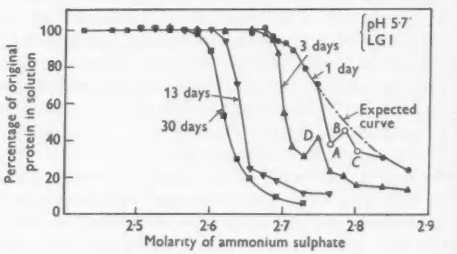

Fig. 8. Solubility curves for LG I at pH 5·7 determined at varying intervals from 1 to 30 days after setting up (i.e. with equilibrium times of 1–30 days).

The curve obtained 1 day after setting up is in general form like the normal curve showing two components, except in the region indicated by the open points where the two points *A* and *C* are seen to lie considerably below the expected curve. The precipitates from those two tubes were found to be almost completely crystalline, the crystals being needle-like and arranged in sheaves. In no other tubes were crystals present to any considerable extent. Thus despite the difficulty of observing under the microscope the proportions of crystalline and amorphous material, there was no doubt that the precipitate from tube *B* was largely of the character described in part (1) of the Results for the pH 4·8 precipitates and contained only a few poorly defined crystals. Consequently, point *B* lies close to the curve (dotted line) expected for precipitates of the bead-like form.

After 3 days the precipitates were all largely of the sheaf-like crystal form, except in tube *D* (the point for which lies above the general curve for 3 days). This tube contained a good deal of amorphous and bead-like material, such crystals as were present being poorly defined.

Solubility changes continued still further until true equilibrium was reached between 20 and 30 days, when the solid phase was found to consist entirely of crystals, now flat diamond-shaped—some arranged in clusters obviously derived from the earlier sheaf-like bundles of needles. At this stage the character of the precipitation curve (filled squares) is quite different from that observed at equilibrium at, for example, pH 4·8. The curve is sigmoid and shows no marked breaks. The precipitation zone is much narrower ($>66\%$ of the protein precipitated with $<2{\cdot}5\%$ change in the salt concentration). This type of curve, in a 'variable solvent' solubility test, may be analogous to the rounded curves obtained in the 'constant solvent' test when mixed crystals of variable composition are present.

The decreases in solubility with time at this pH are thus due to the change from a metastable solid to the stable crystalline form. It is concluded that if the range of salt concentrations over which the alternative form of the solid is stable, is narrow (as the results suggest is the case at pH 5·4), this decreased solubility can lead to the appearance of an additional break in the solubility curves, which suggests erroneously the existence of an additional component in the protein under study. This anomaly should be suspected when precipitation curves are obtained in which any region is found to be unreproducible. It may be recognized by performing the test at several pH values with different lengths of time for equilibrium, and by observing the appearance of the precipitates.

A suggestion which follows from the results presented here is that crystallization should be avoided in the further purification of proteins which have already been crystallized once (cf. Pirie, 1940). The steep precipitation curves with no clear steps, obtained under conditions of crystallization, indicate the danger that all the protein components present in solution, if sufficiently similar, may be included in the highly stable protein crystals if these are once formed. This is probably the explanation of the absence of any change in the component composition of lactoglobulin (noted in part (1) of this paper) following repeated recrystallizations. However, under conditions leading to amorphous precipitates the components come out of solution less abruptly, and to some extent separately, so that further purification by salt fractionation might be accomplished. It should also be noted how small is the change in pH which makes the difference between lactoglobulin being precipitated from these very dilute solutions as an amorphous solid or as crystals.

SUMMARY

1. A method for following attempts at further purifying crystalline β-lactoglobulin was sought.

2. The ultracentrifugal and immunological (precipitation curve determination) methods tried were unsatisfactory as they showed no evidence for inhomogeneity in the sample tested.

3. Electrophoretic analysis of lactoglobulin was subject to such marked anomalies as to be impractical.

4. 'Variable solvent' solubility tests were found most satisfactory, and indicated the presence of two main components (ratio approximately 1:4) in lactoglobulin at pH's 4·5, 4·8 and 5·1.

5. Repeated recrystallizations do not affect this ratio.

6. At pH 5·4 an additional discontinuity occurs in the solubility curves for lactoglobulin associated with a different form of the solid phase (previously amorphous, now crystalline). It is concluded that it does not represent a 'true' additional component in the protein.

7. At pH 5·7 the solubility behaviour of lactoglobulin is markedly time dependent. The results are shown to be due to progressive crystallization, and provide confirmation for the conclusion that the additional break in the curves at pH 5·4 is due to the taking on by the solid phase of a different (crystalline) form stable, at this pH, only over a limited range of salt concentrations.

8. The ways of detecting this anomalous behaviour (when more breaks occur in the solubility curve than there are components in solution) are discussed.

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Phosphorylation of Carbohydrates in Retinal Extracts

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Warburg, Posener & Negelein (1924) first observed that the isolated rat retina together with tumour cells, the grey matter of brain and embryonic tissue possessed comparatively high glycolytic activity. Subsequently, numerous investigations were carried out on the metabolic activity of the isolated retina (e.g. Dickens & Greville, 1933; Laser, 1935). Kerly & Bourne (1939, 1940) and Süllman & Vos (1939), independently, were the first to study glycolysis in retinal extracts. Such extracts were found to convert added glucose, fructose, hexose diphosphate and glycogen into lactic acid; the latter workers also observed the formation of lactic acid from mannose and glucose 1-phosphate. Studies of the effects of dialysis and of activators and inhibitors supported the view that the pathway of lactic acid formation involved the phosphorylating glycolysis which operates in muscle extracts. However, no intermediary phosphate esters were isolated, nor were any of the individual enzymes studied. Kerly (1948) has reported preliminary experiments on a hexokinase in retinal extracts, but again the products of the reaction were not isolated nor identified. Lenti (1939) also studied lactic acid formation in retinal extracts and concluded that phosphorylation was not involved; he failed in all attempts to isolate phosphorylated intermediates (Lenti, 1943).

The present paper provides direct evidence for the occurrence in retinal extracts of the three glycolytic enzymes, hexokinase, phosphohexose isomerase and 6-phosphofructokinase. Some of the characteristics of the hexokinase have been examined and compared with those of yeast hexokinase; certain intermediate phosphoric esters have also been isolated and identified.

METHODS

Materials

AnalaR grade sugars were used without further purification. D-Glucosamine hydrochloride was purified by two recrystallizations from ethanol–water and had a purity of 98–99% on the basis of its nitrogen content. Glucose 1-phosphate (G 1-P) was prepared by the method of Hanes (1940) and was recrystallized as the di-potassium salt. We are indebted to Dr J. Ashby for samples of synthetic D-glucose 6-phosphate (G 6-P) and D-fructose 1:6-diphosphate (Fru 1:6-P) and to Dr C. W. Parr for D-fructose 6-phosphate (Fru 6-P). Adenosine triphosphate (ATP) was made from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948), and inosine triphosphate (ITP) was made from this by the method of Kleinzeller (1942). All the phosphate esters except Fru 1:6-P were prepared as the barium salts and were converted to the soluble sodium salts before use; Fru 1:6-P was prepared and used as the lithium salt, experiments having shown that lithium ions at the concentration employed did not inhibit the phosphorylating enzymes. All solutions were adjusted to pH 7·5 before use.

Preparation of extracts

(*a*) *From retina.* Ox eyes, in batches of one dozen, were fetched on ice from the slaughter house. The retinas were dissected out as soon as possible on arrival in the laboratory and were immediately suspended in 10 ml. ice-cold glass-distilled water. The suspension was ground in a chilled mortar with 2 g. acid-washed sand for 1 min. and was then centrifuged in thoroughly chilled tubes for 5 min. at 1500–2000 *g*. The top, slightly turbid, pink layer was decanted off (or removed with a pasteur pipette) and the residue (a small dark brown layer together with cell debris and sand) was re-extracted with a further 10 ml. glass-distilled water, again centrifuged and the top layer added to the first extract. The combined extracts were dialysed in tightly filled cellophan sacs against running ice-cold glass-distilled

water for 4 hr. The final volume of the extract was 16–18 ml., the dry weight 20–25 mg./ml. and the nitrogen content 2–3 mg./ml.

Attempts to stabilize and purify the hexokinase in retinal extracts were unsuccessful. The addition of 1% (w/v) glucose (cf. van Heyningen, 1942) or of 0·01 M cysteine hydrochloride or sodium sulphide (Bailey & Webb, 1948) to the extracts did not increase their stability. Dialysed or undialysed retinal extracts could not be freeze-dried without large losses of enzyme activity: in many cases complete inactivation resulted. Fractionation with ammonium sulphate gave inconsistent results: an active fraction was precipitated, with little loss of activity, by complete saturation; removal of the precipitate formed by 20–30% saturation increased the activity of the supernatant but higher concentrations of salt caused a distribution of the hexokinase between the supernatant and residue, the activities of both being less than that of the original extract. Low temperature fractions with organic solvents, including butanol (Morton, 1950), resulted in complete loss of activity. Acetone powders, made by pulverizing retinas frozen in a mixture of solid CO_2 and acetone and washing the powder with cold acetone on a Büchner funnel, were stable up to 2 months. The hexokinase activity of aqueous extracts of such acetone powders was always less than that of the crude aqueous retinal extracts (about 60% on a comparable weight basis).

Freshly prepared extracts were used for the experiments reported here, although they were not very stable (hexokinase activity lasted only a few days if the extracts were frozen solid).

(*b*) *From baker's yeast.* In some experiments a partially purified yeast hexokinase preparation was used for comparison with the hexokinase in retinal extracts. The method of Bailey & Webb (1948) was followed as far as their 'stage 4', i.e. autolysis of baker's yeast, extraction in the presence of sulphide, ammonium sulphate precipitation, adsorption on to and elution from calcium phosphate gel and ammonium sulphate fractionation. The final ammonium sulphate fraction was dialysed against several changes of 1% (w/v) glucose solution and was freeze-dried. The preparation was stable for several months when stored *in vacuo* at about 0°. 2–5 μg. dry wt. of this preparation utilized a comparable amount of glucose to 0·1 ml. of dialysed retinal extract.

Tests for enzyme activity

Hexokinase. In most experiments activity was measured by the rate of disappearance of the sugar substrate. In a few cases the reaction was followed by measuring the rate of disappearance of the labile phosphate of ATP; this method is not satisfactory for use with crude extracts as the initial phosphorylated product may undergo further phosphorylation at the expense of more ATP, yielding phosphoric esters which are susceptible to the acid hydrolysis used in the estimation of ATP, so that results are difficult to interpret. A manometric technique, essentially that of Colowick & Kalckar (1941) was also used, particularly for some comparative experiments, but the limitations in the method for crude extracts are similar to those which apply to measurements by changes in labile phosphate.

Except in the manometric method, tests were carried out in open test tubes at 30°. The standard reaction mixture contained: sugar substrate, 0·002 M; $MgCl_2$, 0·01 M; NaF, 0·05 M; ATP, 0·005 M; extract, 0·1 ml. (=200–300 μg. N); veronal buffer pH 7·9, 0·071 M.

The veronal buffer (0·5 ml.) (0·142 M, Michaelis, 1931), together with all the reactants except ATP (total volume 0·9 ml., NaF added last to diminish the risk of precipitation of MgF_2), was equilibrated for 5 min. at 30°, after which the reaction was started by addition of 0·1 ml. 0·05 M ATP. Incubation was usually continued for 10 min., then the reaction was stopped by addition of 1 ml. each of 0·3 M-$Ba(OH)_2$ and 0·3 M-$ZnSO_4$ if sugars were to be estimated, or of 1 ml. 10% trichloroacetic acid if phosphates were to be determined. Control tubes without ATP were included for each sugar substrate; control tubes for phosphate determinations contained ATP but no sugar. In some experiments the total volume of the reaction mixture was increased, but the concentration of all reactants was kept to that stated above.

In the manometric tests the volume of the reaction mixture was increased to 2 ml., the concentrations of the reactants being the same but the relative amount of extract being reduced to 100–200 μg. N per test. Veronal buffer was replaced by 0·02 M-$NaHCO_3$ which was used to buffer the mixture in the main compartment and in the side bulb containing ATP. The flasks were gassed at 30° for 5 min. with a mixture of 95% N_2 and 5% CO_2 and equilibrated until the manometer readings were steady (about 10 min.). Control flasks were always included to correct for gas evolution arising from ATPase action. Rates of gas evolution were uniform over the first 20 min. although there was usually an initial 'jump' in gas evolution when the reaction was initiated, in spite of adequate buffering of the ATP.

Phosphohexose isomerase. Activity was measured by following the disappearance of Fru 6-P. The reaction mixture contained 0·1 ml. of retinal extract, 0·1 ml. 0·02 M Fru 6-P and 0·8 ml. 0·142 M veronal buffer pH 7·5. After incubation for 10 min. at 30°, the mixture was deproteinized by the addition of 1 ml. of 10% trichloroacetic acid and, after centrifuging, the supernatant was examined for residual fructose esters. Control tubes containing an equal amount of a boiled retinal extract were incubated simultaneously.

Phosphofructokinase. Activity was measured by following the changes in acid-hydrolysable phosphate and also manometrically. Conditions were the same as for estimation of hexokinase activity except that Fru 6-P was used as substrate.

Chemical methods

Reducing sugars were estimated colorimetrically by the Somogyi (1945) modification of Nelson's method; standard curves were constructed for fructose, mannose and glucosamine. Fructose both free and combined as Fru 6-P was also estimated, without interference by other reducing sugars, by the method of Bacon & Bell (1948); this method estimates ketose sugars, but as fructose is the only ketose likely to be present it has been assumed that it was in fact fructose that was estimated. Phosphate esters were determined from the increase in inorganic phosphate following hydrolysis for 10 and 30 min. in N-HCl in a boiling-water bath. It was assumed that all the labile phosphate of ATP was liberated in the first 10 min. hydrolysis. Fru 1:6-P is also partially hydrolysed during this period, but as an equal amount (30%) is liberated in the following 20 min. (Lohmann, 1928) a correction can be made, if this ester is present, in calculating changes in the labile phosphate from

ATP. If P_0, P_{10} and P_{30} represent the amount of inorganic phosphate found after hydrolysis for 0, 10 and 30 min. respectively, then after incubation the decrease in labile phosphate from ATP is given by the decrease in

$$(P_{10} - P_0) - (P_{30} - P_{10})$$

(it being assumed further that the amount of phosphate liberated from other esters, e.g. adenylic acid, G 6-P, does not change appreciably during incubation), and any formation of Fru 1:6-P phosphate by the increase in $10/3(P_{30} - P_{10})$. Inorganic phosphate was estimated by the Fiske & Subbarow method (1929).

RESULTS

Hexokinase

D-Glucose, D-mannose, D-fructose and D-glucosamine, but not D-galactose, D-arabinose, D-ribose, D-xylose, nor L-sorbose were utilized by the retinal extracts. Meyerhof & Geliazkowa (1947) and Hele (1950, 1953) have found that extraction of brain and intestine with a modified Ringer solution was necessary in order to demonstrate hexokinase activity towards galactose, but extracts of retina prepared with this solution were still unable to utilize D-galactose, L-sorbose or the above-listed pentose sugars. The pH optimum for the reaction, 7·4–8·6, was similar for all the sugars tested (Table 1). At pH values below 4·0 retinal extracts formed a precipitate and did not utilize any sugars.

The reaction rate for all the sugars utilized by the extracts, was, for any one extract, approximately proportional to the amount of extract added to the incubation mixture over the range 100–500 μg. N (Fig. 1). Rates for the different sugars were similar except that glucosamine was utilized more rapidly (see Table 1). The reaction rate did not vary with sugar substrate concentration over the range 0·001–0·05 M, indicating that retinal hexokinase has a high affinity for the sugars. Competitive utilization was investigated by studying the effect of the addition of other sugars on the utilization of fructose. Glucose, mannose, and glucosamine, but not galactose, inhibited the utilization of fructose. Table 2 illustrates this finding in the case of the binary mixtures of fructose with glucose, and fructose with glucosamine. Weil-Malherbe & Bone (1951) have shown that G 6-P inhibits hexokinase in brain preparations, but in these crude extracts there is no question of inhibition of hexokinase by G 6-P which is rapidly converted into Fru 6-P (see below). G 1-P was not phosphorylated by the extracts and its presence did not interfere with the utilization of glucose.

Kleinzeller (1942) found that ITP could replace ATP as a phosphate donator for yeast hexokinase; this was tested with both retinal and partly purified yeast extracts, but glucose was not phosphorylated in the presence of either extract by ITP. The reason for the difference from Kleinzeller's results is not apparent.

Earlier work with retinal extracts (Kerly & Bourne, 1940; Süllman & Vos, 1939) showed that Mg^{2+} was necessary for glycolytic activity, but the effect of other divalent ions was not investigated. The effect of a number of divalent ions was studied in 12–18 hr. dialysed retinal extracts, and for comparison, in partially purified yeast extracts. The incubation period was increased to 30 min. as the retinal extracts had lost 50% of their activity during the extra period of dialysis. Since heavy metals form chelation complexes with veronal, the buffer was replaced by 0·1 M *N*-ethyl morpholine

Table 1. *Effect of* pH *on utilization of sugars by retinal extract*

0·1 ml. extract incubated 10 min. at 30°.

	Sugar used (μmole/mg. N) when substrate was			
pH	Glucose	Fructose	Mannose	Glucosamine
4·6	1·7	2·3	1·5	0·3
5·8	2·4	2·5	2·1	3·4
7·4	3·6	4·8	3·5	7·3
8·6	3·7	3·7	4·7	7·2

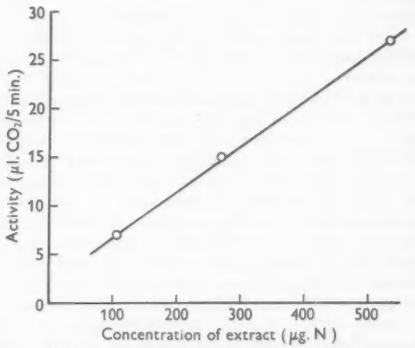


Fig. 1. Effect of concentration of extract (measured as μg. N) on hexokinase activity of retinal extract; μl. CO_2 liberated in 5 min. from extract incubated at 30° with 0·002 M glucose.

Table 2. *The effect of glucose and glucosamine on the utilization of fructose by retinal extracts*

0·1 ml. extract incubated 10 min. at 30°, pH 7·9. Fructose concn. 0·002 M.

	Glucose added		Glucosamine added	
Concn. of glucose or glucosamine (M)	Fructose used (μg.)	Inhibition (%)	Fructose used (μg.)	Inhibition (%)
0	150	—	142	—
0·002	0	100	0	100
0·001	0	100	42	71
0·0005	50	67	65	51
0·0002	95	37	105	26
0·0001	145	0	115	20

adjusted to the required pH with 0·1 N-HCl. Hexokinase activity was not influenced by the change in buffer. Fluoride was omitted to avoid any effects which might have arisen from varying solubilities of the metal fluorides. Manganese, and to a much less extent cobalt, but not calcium, beryllium, copper, zinc, mercury, or ferrous iron, could replace magnesium (see Table 3). All the metal ions were added as the chlorides, except ferrous iron which was in the form of ferrous ammonium sulphate; they were all tested at a concentration of 0·01 M, that which was found to be optimum for Mg and Mn (Table 4). Bailey & Webb (1948), using yeast extract, found that calcium did not antagonize magnesium; this effect was tested with retinal extracts and a similar result was obtained, i.e. Ca^{2+} does not antagonize Mg^{2+} in retinal extracts.

An attempt was made to identify the products of the action of retinal hexokinase on the different sugar substrates. Test mixtures incubated with glucose, mannose or fructose were deproteinized by trichloroacetic acid and by barium hydroxide–zinc sulphate. Filtrates obtained by the former method of deproteinization gave a colour when tested with the Bacon & Bell ketose reagent, but those deproteinized by the latter method showed no colour, indicating that a fructose phosphate had been formed. When glucosamine was used as substrate no ketose colour appeared in either filtrate. The experiments were repeated using a larger volume of reaction mixture and the trichloroacetic acid filtrates were fractionated with barium (LePage, 1949).

When glucose or fructose was used as substrate, the ketose reaction was found in the barium-insoluble fraction, but only weakly in the barium-soluble fraction, indicating that the predominant ester formed was Fru 1:6-P. Studies of the rates of hydrolysis of the phosphate esters formed after incubation of the extract with glucose further confirmed the formation of Fru 1:6-P, whereas simultaneous experiments with glucosamine as substrate gave no indication of formation of a readily hydrolysable ester such as Fru 1:6-P (Table 5). Calculation showed that more than one labile phosphate group from ATP was utilized per mole of glucose disappearing, but approximately one mole per mole of glucosamine. After incubation of the extract with glucosamine a phosphate ester was found in the barium-soluble–alcohol-insoluble fraction, indicating a hexose monophosphate as the product of

Table 3. *The effect of divalent cations on glucose utilization by yeast and retinal hexokinase*

0·1 ml. 18 hr. dialysed extract incubated, without addition of NaF, 30 min. at 30°, pH 7·9. Metal-ion concn. 0·01 M.

Cation added	μg. Glucose used with enzyme from: Yeast	Retina
Mg	230	160
Mn	220	140
Co	50	30
Ratio of activity		
Mn/Mg	0·96	0·88
Co/Mg	0·22	0·19

Table 4. *The effect of concentration of* Mg *and* Mn *on glucose utilization by retinal extracts*

0·1 ml. 18 hr. dialysed extract, incubated without addition of NaF, 30 min. at 30°, pH 7·9.

Concn. of added ion (M)	μg. Glucose used in presence of: Mg	Mn
0·02	130	—
0·01	140	130
0·005	120	90
0·001	90	50
0·0005	60	30

Table 5. *Utilization of glucose and ATP and formation of Fru* 1:6-P *in retinal extracts incubated with glucose and glucosamine*

0·1 ml. extract incubated for 30 min. at 30°, pH 7·9. P_0, P_{10} and P_{30} represent μg. inorganic phosphate (as phosphorus) found after hydrolysis for 0, 10 and 30 min. respectively. See text for method of calculating decrease in labile phosphate from ATP and formation of Fru 1:6-P.

	Extract incubated: Without sugar (control)	With glucose	With glucosamine
$P_{10} - P_0$	126	92	79
$P_{30} - P_{10}$	10	21	8
$(P_{10} - P_0) - (P_{30} - P_{10})$	116	71	71
Decrease in labile phosphate from ATP	—	45	45
Formation of Fru 1:6-P phosphate	—	37	—
μg. Sugar utilized (as glucose)	—	160	300
Molar ratio: Labile phosphate/sugar	—	1·64	0·87

Table 6. *Acid hydrolysis of glucosamine phosphate*

$1{\cdot}6 \times 10^{-3}$ M ester hydrolysed at 100° in N-HCl.

Time (min.) ...	30	60	120	
Phosphate liberated (% total)	5·4	9·4	20·0	
$10^5 K$*	3·2	2·6	3·1	Mean, 3·0

* Dissociation constant calculated from the equation for a first-order reaction: $K = 1/t \log_e a/(a-x)$, where t = time in sec., a = initial concn. and $(a-x)$ concn. at time t.

hexokinase action on this sugar. Evidence that this compound was a phosphate ester and not a glycoside came firstly from the finding that it was a reducing sugar (the reducing value being increased after acid hydrolysis), and secondly from its stability to hydrolysis in acid solution (Table 6). By analogy with the work of Brown (1951) on brain extracts it is likely to be glucosamine 6-phosphate.

Phosphohexose isomerase

The results reported in the previous section indicate that phosphorylation continues to the Fru 1:6-P stage. By analogy with other glycolytic systems, this requires the presence of phosphohexose isomerase to convert the initial product of the reaction, presumed to be G 6-P, into Fru 6-P which is then phosphorylated to Fru 1:6-P. Direct evidence for the presence of this enzyme (phosphohexose isomerase) in crude retinal extracts was obtained in two ways, first by following the change in fructose colours when Fru 6-P was incubated with extract but no ATP (Table 7), and secondly by showing that G 6-P reacts in a similar manner to Fru 6-P when added to the test system for phosphofructokinase (Fig. 2). The enzyme must be more active than hexokinase since Fru 6-P disappeared rapidly even in extracts diluted 100 times, a dilution at which no hexokinase activity was apparent.

Phosphofructokinase

The identification of Fru 1:6-P as a product of the incubation of glucose with crude retinal extracts, and the direct demonstration of a hexokinase and a phosphohexose isomerase in the extracts indicate that Fru 1:6-P is formed by the consecutive action of these two enzymes and a phosphofructokinase. Direct evidence for the presence of this last enzyme was obtained by following the changes in hydrolysable phosphate when either G 6-P or Fru 6-P was added to the reaction mixture. Approximately 1 mole of Fru 1:6-P was formed for each mole of ATP utilized (Table 8). Utilization of G 6-P and Fru 6-P was also followed manometrically. There was no significant gas evolution without addition of ATP, indicating the absence of an active phosphatase hydrolysing either of these esters. The rates of gas evolution were always greater than that observed with glucose (Fig. 2) implying that the hexokinase reaction is the rate limiting step in the formation of Fru 1:6-P from glucose. With aged extracts, phosphofructokinase activity was lost more rapidly than that of hexokinase. In acetone-powder extracts also, phosphofructokinase activity was reduced relative to that of hexokinase (Fig. 3), indicating that phosphofructokinase is more labile than hexokinase.

Table 7. *Disappearance of Fru 6-P in retinal extracts incubated without ATP*

Extract containing 0·002 M Fru 6-P incubated 10 min. at 30°, pH 7·5; control = boiled extract.

Concn. of enzyme (μg. N/ml.)	Fructose remaining after incubation: Colorimeter reading	% Control
Control: 250	20	—
250	6	30
25	6	30
2·5	8	40

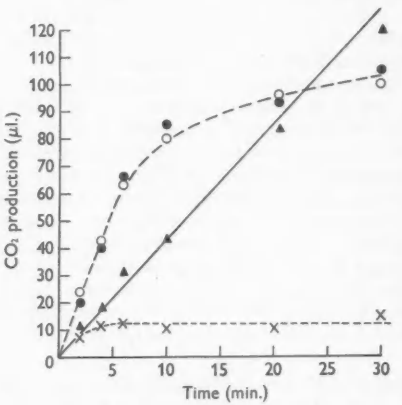


Fig. 2. Comparison of rates of activity of phosphofructokinase and hexokinase in retinal extract: μl. CO_2 produced by 0·1 ml. extract incubated at 30°. × – – – ×, ATP only, no substrate; ▲—▲, 0·002 M glucose; ●– – –●, 0·002 M G 6-P; ○– – –○, 0·002 M Fru 6-P.

Table 8. *Utilization of ATP and formation of Fru 1:6-P in retinal extracts incubated with Fru 6-P*

0·1 ml. extract incubated 10 min. at 30°, pH 7·9. P_0, P_{10} and P_{30} represent μg. inorganic phosphate (as phosphorus) found after hydrolysis for 0, 10 and 30 min. respectively. See text for method of calculating decrease in labile phosphate from ATP and formation of Fru 1:6-P.

	Extract incubated: Without Fru 6-P (control)	With Fru 6-P
$P_{10} - P_0$	155	136
$P_{30} - P_{10}$	22	45
$(P_{10} - P_0) - (P_{30} - P_{10})$	133	91
Decrease in labile phosphate from ATP	—	42
Formation of Fru 1:6-P phosphate	—	77
Molar ratio: labile P/Fru 1:6-P	—	1·09

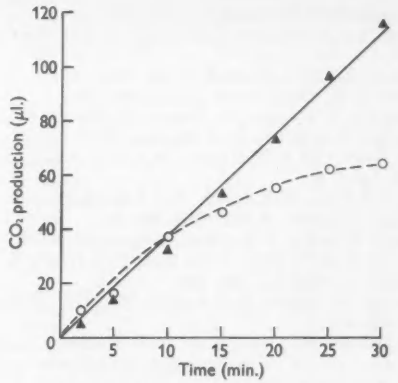
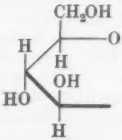


Fig. 3. Comparison of rates of activity of phosphofructokinase and hexokinase in an aqueous extract of acetone-dried retinas. Extract (0·1 ml.) containing 400 µg. N, incubated at 30°. ▲—▲, 0·002 M Glucose; ○- - -○, 0·002 M Fru 6-P.

DISCUSSION

The hexokinase in retinal extracts is analogous to the hexokinase from yeast in its metal ion requirements and substrate specificity. The inhibition of fructose utilization by glucose and glucosamine suggests that the three sugars are substrates of a common hexokinase, as in yeast, in contrast to the separate gluco- and fructo-kinases which exist in muscle (Slein, Cori & Cori, 1950) and in liver (Vestling, Mylroie, Irish & Grant, 1950). It is of interest that all the sugars utilized, including glucosamine, contain the partial structure shown to



be essential for all substrates of yeast hexokinase (Gottschalk, 1950), whereas glucose 1-phosphate, which also contains this structure, is not attacked. In muscle a specific enzyme has been found which phosphorylates G 1-P (Paladini, Caputto, Leloir, Trucco & Cardini, 1949). It may be noted that 2-deoxyglucose, another sugar containing the above structure, has recently been shown to be phosphorylated by yeast (Cramer & Woodward, 1952) and by brain hexokinase (Sols & Crane, 1953). The hexokinase from intestinal mucosa may be fundamentally different from that of yeast and retina since it phosphorylates sugars which do not have this structure, e.g. 3-methylglucose (Csaky, 1953), galactose and xylose (Hele, 1953) none of which are phosphorylated by yeast or retinal hexokinase.

It is significant that in retinal extracts glucosamine cannot be metabolized beyond the stage of the monophosphate, although fructose 6-phosphate and glucose 6-phosphate are rapidly metabolized. Very little is known of the metabolism of glucosamine. Harpur & Quastel (1949) first reported its phosphorylation in brain extracts and further showed that *N*-acetyl glucosamine, which was not phosphorylated, inhibited the phosphorylation of glucosamine, fructose and glucose by the brain preparation, and Brown (1953) has shown that glucosamine 6-phosphate is slowly converted into glucosamine 1-phosphate by rabbit muscle extracts and by purified phosphoglucomutase. It is of interest that liver and brain extracts and the lactose yeast *Saccharomyces fragilis*, which are capable of phosphorylating galactose, also phosphorylate the corresponding amino sugar galactosamine (Cardini & Leloir, 1953).

SUMMARY

1. The presence of hexokinase in retinal extracts has been demonstrated. In the presence of ATP it phosphorylates glucose, fructose, mannose and glucosamine. Magnesium, manganese and to a smaller extent cobalt activate the enzyme in dialysed extracts.

2. Both glucose and glucosamine inhibit the utilization of fructose, implying that all three sugars are substrates of a common hexokinase.

3. Glucosamine is converted into a monophosphate derivative which is not further metabolized by the extracts; the other sugars yield fructose 1:6-phosphate.

4. Direct evidence for the presence of phosphohexose isomerase and of phosphofructokinase is also presented.

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Monoamino Monocarboxylic Acid Content of Preparations of Herbage Protein

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The similarity in amino acid composition of numerous 'representative' leaf proteins and of the constituent cytoplasmic and chloroplastic fractions is fairly well established but some doubt remains as to the degree of similarity, variations with age and species having been reported (review by Lugg, 1949).

Earlier analyses were rendered difficult by the dearth of suitable techniques and have, in general, been confined to those amino acids which are readily determined by means of some characteristic group in the molecule. Unfortunately, it is often these substances that are most labile under the conditions of hydrolysis.

Before investigating the fate of amino acids liberated by proteolysis in herbages under conditions of starvation (e.g. wilting and ensilage) it was essential to know whether the residual-protein fractions were of constant composition or whether it would be necessary to undertake analysis in every case. The method of Kemble & Macpherson (1954), which was developed primarily for the amino acid analysis of plant extracts and which was to be used for the proteolytic investigations, was therefore applied to the analysis of protein-rich fractions extracted by several methods from a variety of herbages.

EXPERIMENTAL

Source and extraction of proteins

Protein A. The protein was extracted from perennial rye grass (S. 24 hay strain) by the method of Crook (1946), which entails repeated grinding with sodium borate buffer at pH 8·0. The grass was senescent, being cut in December. 62·5% of its total protein-N was extracted, as material having N 10·8% of dry matter.

Protein B. Mixed grass from permanent pasture was macerated in 0·05M borax, filtered through paper pulp and precipitated at pH 4·5. The protein, after heat coagulation, was washed with ethanol and ether. 41·2% of the grass protein-N of the grass was obtained as material having N 14·5% of dry matter.

Protein C. Young lawn grass (height 4–6 in.) was macerated with a solution of borax (2%, w/v). After filtration through muslin the solution was brought to pH 4·5 and the protein coagulated by heating to 90°. 38·2% of the total protein-N was obtained; N, 8·9% of dry matter.

Protein D. Young perennial rye grass (S. 24 hay strain) was cut in May (height 4–6 in.). It was finely minced and the sap filtered by suction through muslin and then through paper. The protein was coagulated by heating to 90° at the pH of the sap (6·3). It was washed with hot water, ethanol and ether and represented 32·8% of the fresh grass protein-N; N, 13·4% of dry matter.

Protein E. A sample of alsike clover was cut before flowering and extracted as described for protein *A*. 39% of the clover protein-N was obtained as material having N 14·3% of dry matter.

Protein F. A similar grass sample to that used for protein *C* was allowed to wilt in the laboratory for 48 hr. In this time 10% of the protein had been degraded. The remainder was extracted as described for protein *C*. 37% of the total protein-N was extracted; N, 9·0% of dry matter.

Protein G. An identical sample of sap to that from which protein *D* was obtained was bottled, covered with a thick layer of liquid paraffin and placed in an incubator at 30°. These conditions simulated the ensilage process (Macpherson, 1952) and the pH dropped rapidly to 3·9. After

Table 1. *Monoamino monocarboxylic acid content* of seven herbage proteins*

	N as % total N							
Preparation	*A*	*B*	*C*	*D*	*E*	*F*	*G*	Barley (Yemm & Folkes, 1953)
Glycine	6·8	6·4	6·9	6·7	7·0	6·9	6·7	6·7–6·9
Serine†	4·2	4·1	4·2	4·0	3·9	4·0	3·6	3·3–3·5§
Threonine†	4·3	4·3	4·4	4·5	4·2	4·2	3·9	4·0§
Hydroxyproline					Absent			
Alanine	5·9	5·7	5·8	5·9	5·9	5·8	6·0	7·9–8·3
Proline	3·5	3·6	3·7	3·6	3·8	3·6	3·4	3·9–4·1
Tyrosine‡	1·7	1·7	1·5	1·7	1·6	1·6	1·7	2·2–2·3
Valine	4·4	4·8	4·7	4·8	4·7	4·5	4·5	4·9–5·1
Phenylalanine	3·5	3·6	3·5	3·6	3·3	3·5	3·6	3·0–3·2
Isoleucine	3·4	3·3	3·4	3·4	3·5	3·4	3·5	4·2–4·7
Leucine	5·7	5·3	5·9	5·5	5·7	5·3	5·4	5·8–6·0
Insoluble humin	4·2	1·6	3·2	4·9	1·1	6·1	3·9	2·4–4·3
Ammonia (formed on hydrolysis)	8·7	8·8	8·8	8·9	7·5	9·4	8·8	5·8–6·0

* Individual methionine values (0·2–0·8) are not recorded due to known heavy losses by this method of hydrolysis (Bailey, 1937).

† Corrected for hydrolysis losses (Rees, 1946).

‡ Minimum as from acid hydrolysate.

§ Uncorrected for hydrolysis losses.

15 weeks a precipitate (coagulated protein) was removed by filtration. Determination of the soluble nitrogen showed that 33% of the original protein had been degraded. The precipitate was washed as described for protein *D*; N, 9·7% of dry matter.

Analysis

Hydrolysis. Samples (250–350 mg.) of protein were boiled under reflux for 24 hr. with 500 ml. 6N-HCl and taken to dryness *in vacuo*. The water-soluble material was separated from the insoluble humin by filtration and used for analysis.

Nitrogen determinations. These determinations were carried out by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943).

Amino acid determinations. Analysis was carried out by the method of Kemble & Macpherson (1954).

RESULTS AND DISCUSSION

The proteins examined were obtained from individual species of different plant families (proteins *A*, *D* and *E*) and from uncontrolled mixtures of grasses (proteins *B* and *C*). The parent plants differed in stage of growth and the proteins varied greatly in purity and method of extraction. The latter variation implies a difference in the cytoplasmic/chloroplastic ratio, this being relatively high for *D* and low for *A*.

In spite of these variables, no significant difference could be detected in the monoamino monocarboxylic acid content of the proteins (Table 1). Furthermore, the results, other than for isoleucine and alanine, are in good agreement with those obtained by microbiological analyses of barley proteins (Yemm & Folkes, 1953).

The humin figures are not high considering the impurity of some of the preparations and this is probably due to the great dilution of the protein during hydrolysis (HCl: protein = 300:1, w/w). Even so, much destruction of methionine and probably some destruction of tyrosine has occurred.

In view of these results, it would seem reasonable to assume a consistent monoamino monocarboxylic acid composition for representative herbage proteins. However, when following the proteolysis which is a normal accompaniment of plant starvation, knowledge of the original protein composition is of no value unless it is also known if its breakdown is uniform. Hence two additional protein preparations (*F* and *G*) were analysed after extraction from grasses undergoing different types of starvation (wilting and ensilage respectively). Protein *F* gave an analysis typical of the fresh herbage proteins but protein *G* showed small but measurable diminutions in the values for the hydroxyamino acids, serine and threonine (Table 1).

This may imply some preferential release of these two amino acids. However, the protein had remained in contact with a solution of pH 3·9 for 15 weeks and since the hydroxyamino acids are the most labile under acid conditions, the possibility of some purely chemical degradation cannot be excluded. Apart from these two slight discrepancies the analysis was typical of a fresh herbage protein.

These findings demonstrate that protein breakdown during starvation is uniform in that the amino acid composition of the residual protein is virtually the same as that of the original.

SUMMARY

1. Application of a new and accurate method of analysis has not shown any significant difference in the monoamino monocarboxylic acid composition of representative herbage proteins.

2. Analysis of a protein extracted from wilted grass was not significantly different from that of fresh grass.

3. Analysis of a protein extracted from ensiled grass showed significant differences only in its content of serine and threonine, and these differences were small.

We wish to express our appreciation of the interest shown by Prof. E. L. Hirst, F.R.S. The work forms part of a programme of research on crop conservation sponsored by the Agricultural Research Council.

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Liberation of Amino Acids in Perennial Rye Grass During Wilting

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Proteolysis during starvation of plant material gives rise to an increase in peptide, free amino acids and amides. The amides appear in amount greater than that contained in the protein and must incorporate nitrogen liberated by deamination of amino acids. Thus some of the amino acids released by proteolysis are metabolized further and do not appear in the calculated amount. For example, Wood & Cruikshank (1944) have shown cystine, glutamic acid, arginine, tyrosine and tryptophan to be oxidized in that order of rapidity.

No evidence is available concerning the remainder of the amino acids and the investigation has been extended to include the majority of the monoamino monocarboxylic acids.

EXPERIMENTAL

Analysis

Total nitrogen. The total nitrogen (TN) is that nitrogen in the plant determinable by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943). All results are expressed as a percentage of this TN.

Soluble nitrogen (SN). This is the fraction of the TN that is soluble in boiling water. Three successive extractions of 250 g. fresh material were made, each by 1 l. of boiling water.

Volatile base. The volatile base was determined by distilling a portion of the SN extract at pH 10·5 into standard acid.

Total amide. Asparagine and glutamine were hydrolysed by making a sample of the SN extract N with respect to H_2SO_4 and boiling under reflux for 3 hr. The increase in volatile base measures the NH_3 formed (Vickery, Pucher, Clark, Chibnall & Westall, 1935).

Glutamine. A sample of the SN extract was boiled under reflux at pH 6·5 for 2 hr. (Vickery *et al.* 1935). The increase in volatile base measures the NH_3 formed by the hydrolysis of glutamine. Some hydrolysis must have occurred during extraction but the resulting error cannot be great.

α-Carboxyl nitrogen. This was determined on a sample of the SN extract by the ninhydrin–CO_2 titrimetric procedure of Van Slyke, MacFadyen & Hamilton (1941).

Peptide. A sample of the SN extract (approximately 1 mg. N/ml.) was treated with 3 parts by vol. of ethanol. The resulting precipitate was removed by centrifuging and the N content of the precipitate determined. It is not claimed that this precipitate necessarily contains all the peptide present, but at least no α-carboxyl nitrogen is precipitated under these conditions and the determination is of value for comparison purposes.

Monoamino monocarboxylic acids. The ethanol was distilled from the supernatant solution obtained during the previous determination and a sample containing about 30 mg. N was subjected to ionophoresis. The neutral amino acids were estimated by the method of Kemble & Macpherson (1954*a*).

First wilting experiment

Young perennial rye grass (S. 24 hay strain; moisture content 88·3%; N, 4·56% of dry matter) was cut on 17 June 1953. The grass was 6–8 in. in height.

Six 250 g. samples were taken and the first was extracted immediately with boiling water. The remaining five samples were spread thinly on a laboratory bench and the SN was extracted after 1, 2, 3, 5 and 8 days respectively. The air temperature during the experiment was 14–18°.

Second wilting experiment

A mature sample of perennial rye grass (moisture content, 81·0%; N, 2·07% of dry matter) was cut on 28 October 1953. The grass (12–18 in.) was an aftermath and contained no flower heads.

Two samples (69·6 g. each) were taken. The first was allowed to wilt as before. The second was placed in a tall bottle and a slow stream of air saturated with water vapour was passed through the bottle continuously.

The SN was extracted from each after 3 days.

RESULTS

The results of the first wilting experiment are shown in Figs. 1–4. The fresh grass gave 0·5% of TN as peptide precipitable from the SN fraction. For wilted grass this figure rose to 2·5 and 3·3% after 1 and 8 days respectively. The values for the individual amino acids are compared with the values expected assuming uniform protein breakdown. These latter values are calculated from the expression

$$A_f + A_p\left(\frac{S_n - S_f}{100}\right),$$

where A_f is the free amino acid content of the fresh grass, A_p is the amino acid content of the protein and S_f and S_n are the soluble nitrogen fractions (% of

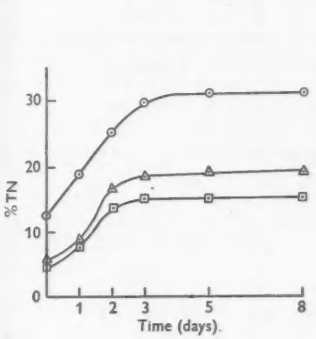

Fig. 1.

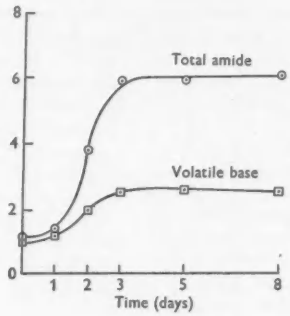

Fig. 2.

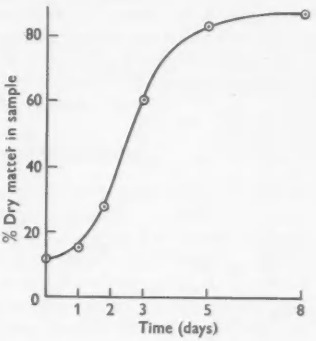

Fig. 3.

Figs. 1–3. Fig. 1. Soluble nitrogen (⊙), α-carboxyl N (⊡), and α-carboxyl N + volatile base + half amide N (△), formed in wilting grass (Expt. 1). Fig. 2. Formation of amide and volatile base in wilting grass. Fig. 3. Increase in dry-matter content of grass during wilting.

Table 1. *Proteolysis in two identical samples of cut grass kept 3 days under different conditions of humidity*

Original moisture content, 81·0%. TN 2·07% of dry wt.

	Wilting		Moist wilting*	
Original weight (g.)	69·6		69·6	
Weight after 3 days (g.)	19		68·6	
	N as % TN			
	Found	Expected	Found	Expected
Soluble N	30·6	—	38·6	—
Volatile base	0·5	—	1·5	—
Glutamine†	2·6	—	3·6	—
Asparagine†	4·8	—	10·4	—
α-Carboxyl N (less half amide N)	10·5	—	9·0	—
Glycine	0·09	1·15	0·15	1·68
Serine	0·81	1·01	1·24	1·34
Threonine	0·48	0·74	0·61	1·06
Hydroxyproline	Absent			
Alanine	0·41	1·26	0·26	1·73
Proline	3·91	0·85	0·53	1·13
Tyrosine	0·08	0·50	0·12	0·68
Valine	0·58	0·85	0·76	1·22
Methionine	0·08	0·24	0·03	0·38
Phenylalanine	0·43	0·58	0·26	0·93
Leucine	0·20	0·84	0·20	1·32
Isoleucine	0·36	0·53	0·36	0·84
Peptide N (precipitable in aq. ethanol, 75% v/v)	3·0	—	4·0	—

* See text for definition.

† The total amide N and asparagine/glutamine ratios are in excellent agreement with those recorded in a similar experiment (Macpherson, 1952).

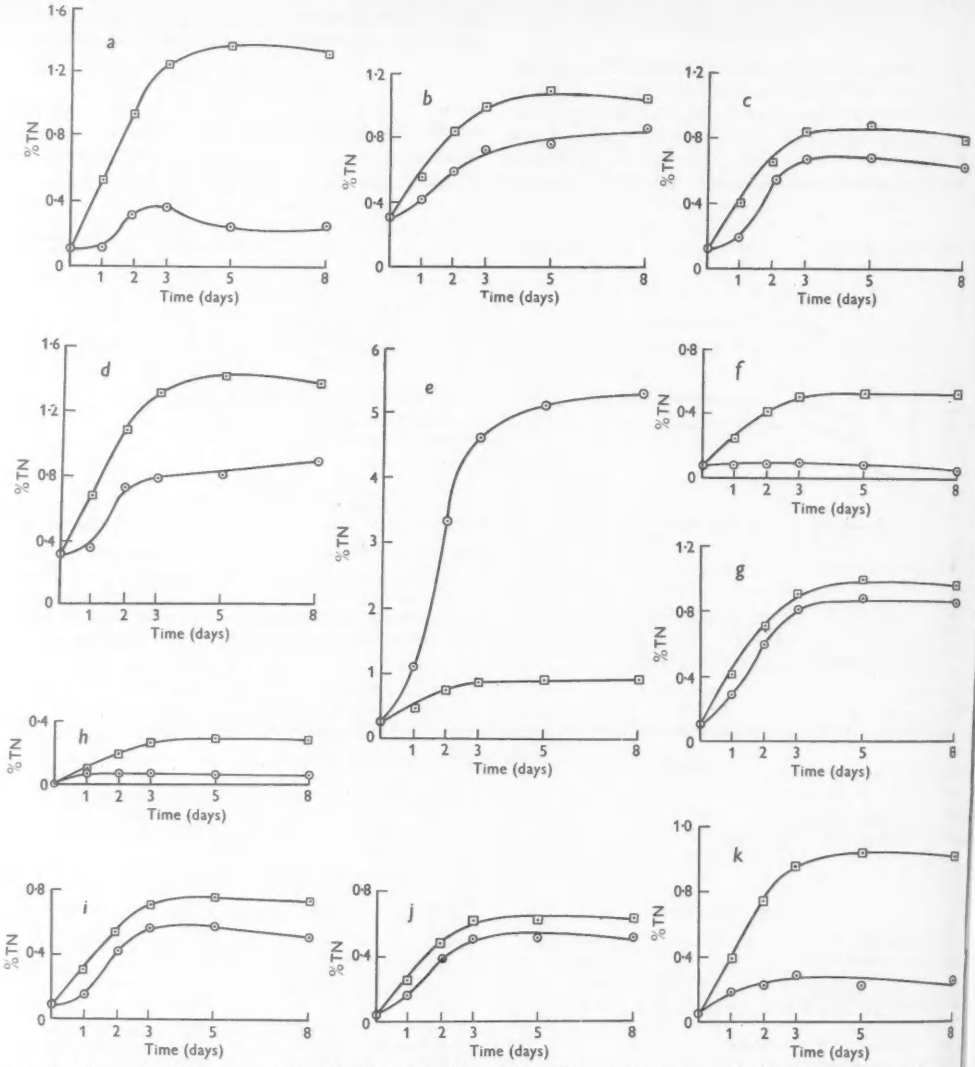

Fig. 4. Comparison between amino acids formed during wilting (⊙) and the values calculated assuming uniform breakdown of the protein (⊡). (*a*) Glycine; (*b*) serine; (*c*) threonine; (*d*) alanine; (*e*) proline; (*f*), tyrosine; (*g*), valine; (*h*), methionine; (*i*), phenylalanine; (*j*), isoleucine; (*k*), leucine.

TN) of the fresh grass and *n*th day's wilted grass respectively.

Values for the amino acid content of the protein are taken from Kemble & Macpherson (1954*b*) except for tyrosine (2·4 % of TN) and methionine (1·5 % of TN) (review by Lugg, 1949).

The results of the second wilting experiment are contained in Table 1.

DISCUSSION

Fig. 1 shows the overall course of proteolysis during the first wilting experiment. A considerable fraction of the soluble nitrogen is obviously not determined as amino acid, volatile base or amide. However, little or no increase in these undetermined substances took place during wilting as the small apparent

increase is accounted for by the increase in alcohol-precipitable peptide. The lag in α-amino acid liberation as compared with the increase in soluble nitrogen during the first 2 days is paralleled by the observed results for many of the individual amino acids (Fig. 4) and shows that the formation of free amino acid occurs via peptides rather than as a direct degradation of the protein molecule. This is confirmed by the sharp rise in the amount of soluble nitrogen precipitable by aqueous ethanol after the grass had wilted for 1 day.

The formation of amide was greatest during the second and third days after cutting (Fig. 2), as had been observed in detached barley leaves (Yemm, 1937), but cessation of nitrogen metabolism was virtually complete after 3 days, by which time the moisture content of the grass had fallen to approximately 40 % (Fig. 3).

Determinations of the individual amino acids in fresh grass (Fig. 4) were in agreement with the semiquantitative observations of Synge (1951). During the ensuing wilting all the amino acids, with the exception of proline, were found in amounts less than those calculated from protein degradation. The deficit was large for glycine, leucine and tyrosine and moderately large for alanine. This general pattern was also observed with a more mature grass (Table 1). It has been shown that the breakdown of protein during wilting is uniform (Kemble & Macpherson, 1954*b*) and so these deficiencies must be a measure of the rate at which the amino acids are metabolized after liberation.

Proline was the only amino acid which occurred in excess of expectation and this excess was very great. After 8 days the amount of free proline was 50 % in excess of that present in the original protein, even though only one-fifth of this had been degraded (Fig. 4*e*). Confirmation of this unexpected result was provided by the intensity of the distinctive yellow spot given by proline on a ninhydrin-sprayed chromatogram.

In a similar experiment carried out with mature grass, the pattern of glycine, leucine, tyrosine and alanine disappearance and of proline synthesis again emerged (Table 1). However, an identical sample of this grass which was not allowed to lose moisture during starvation ('moist wilting') did not synthesize proline. Thus some degree of desiccation would appear to be essential for proline formation.

There is little known concerning the synthesis of proline in higher plants but its formation in the rat can occur via glutamic acid (Sallach, Koeppe & Rose, 1951), synthesis of which, or at least its amide, can take place in starving plants. However, proline formation during wilting does not appear to be a simple matter of glutamine conversion, as the large difference in total amide between the wilting and moist wilting procedures is due almost entirely to the absence of asparagine (Table 1). Nevertheless, the accumulation of proline is comparable in magnitude with the amount of amide synthesized during moist wilting, especially if the comparison is made on a molecular basis rather than on nitrogen content, and it is possible that, under conditions of desiccation, proline synthesis undertakes a similar metabolic role to that normally provided by amide formation. In this respect it should be noted that there is no pronounced difference in the pattern of amino acid disappearance during the two wilting procedures (Table 1). Such a difference might have been expected if proline and amide synthesis were totally divorced from each other.

SUMMARY

1. The various groups of nitrogenous compounds formed by proteolysis in wilting grass have been estimated and it has been shown that all the nitrogen from the degraded protein reappears as α-amino acid, volatile base, amide or peptide.

2. Many of the free amino acids have been determined and the results compared with those expected from the extent of protein breakdown. Except for proline, all the amino acids were present in amounts smaller than calculated and these deficits varied greatly.

3. Proline was found in wilting grass in amounts greatly in excess of expectation but no synthesis occurred when the grass was not allowed to lose moisture during starvation. The possibility of a connexion between this synthesis and the known formation of amide during starvation is discussed.

We wish to express our appreciation of the interest shown by Prof. E. L. Hirst, F.R.S. The work forms part of a programme of research on crop conservation sponsored by the Agricultural Research Council.

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The Properties of a Complex of the Mucopolysaccharide and Proteins of the Cornea

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It has been established that the acid mucopolysaccharide of the cornea is always extracted in combination with protein (Woodin, 1952). Several complexes are produced but one of them, the phenol-insoluble mucoid, is particularly stable, and the properties of this substance have been investigated further to determine if the protein component is a collagen derivative and in an attempt to identify the linkage between the protein and the mucopolysaccharide. In approaching the latter problem it was decided to use methods which might reveal electrostatic linkage before looking for covalent bonds.

Although there is little information on the mucoids formed in connective tissues from mucopolysaccharides which contain ester sulphur, two classes can be distinguished (Kellgren, 1952; Meyer, 1953). The mucoid from cartilage, which was described by Partridge (1948), contains a collagen derivative which is separated at pH 11 or on electrophoresis, whilst those present in tendon, heart valves and aorta do not contain collagen and are stable under these conditions (Meyer, 1953). It will appear from the results to be described here that the corneal mucoid is of the latter class.

The mucins formed, in acid solution, from chondroitin sulphate and proteins were shown by Meyer, Palmer & Smyth (1937) to be salts formed from the acid groups of the mucopolysaccharide and the basic groups of the protein, but the interaction in the mucoids which are stable at pH values where both components would have a net negative charge has not been considered before. Interaction under physiological conditions will be of this sort and so is fundamental to the understanding of the structure of the ground substance of connective tissues.

A preliminary account of this work has appeared (Woodin, 1953).

METHODS

Preparation of material

The phenol-insoluble mucoid. This was prepared from the water-insoluble sections of ox corneas by the methods described previously (Woodin, 1952). This mucoid represents 2·2% of the dry wt. of the cornea and contains 32% of the ester sulphate. Some physical measurements were made on samples of the phenol-insoluble mucoid after precipitation from solution in formamide. The phenol-insoluble mucoid was suspended in formamide; all but about 10% dissolved to give a clear solution. Ethanol (2 vol.) was added and the precipitate collected by centrifuging, suspended in water, dialysed in cellophan against water and the solution freeze-dried. This material represented 1·8% of the dry wt. of the cornea. A small variable amount of the phenol-insoluble mucoid was insoluble after freeze-drying and some experiments were made on a sample which had not been dried but was dialysed against the solvents used in the physical measurements. The properties of these solutions did not differ from those of solutions of dried mucoid.

The mucopolysaccharide. This was prepared from the phenol-insoluble mucoid by digestion with trypsin in the manner already described (Woodin, 1952), except that after the digestion the solution was dialysed against NaCl ($I = 0{\cdot}18$), sodium phosphate buffer ($I = 0{\cdot}02$, pH 7), and the fast component separated on electrophoresis. There were two components, of which the slower moved but little from the initial position of the boundary. The yield of the mucopolysaccharide depends on the length of the electrophoretic separation, but as only one component has an appreciable mobility it is considered that this component is representative of all the material which contains ester sulphur in the mucoid.

Gelatin. Gelatin from the cornea was prepared from the sections which had been extracted with water and 10% (w/v) aqueous $CaCl_2$. The residues were washed free from $CaCl_2$, suspended in water in a sealed tube and heated at 110° for 18 hr. The insoluble material was centrifuged off and the solution kept frozen. This substance contained 16% N; its homogeneity was not investigated. It represented 60% of the dry wt. of the cornea.

The yields of these materials are based on the dry wts. obtained by heating at 105°. The corneal sections were weighed before and after extraction and samples taken for the determination of dry weight and inorganic material. The volumes of the solutions of the mucoids and gelatin, obtained from known weights of the corneal sections, were noted and samples dialysed against 0·001 N-HCl and then distilled water, the volumes noted again, and samples dried at 105°. It was assumed that the inorganic material was removed by the dialysis.

Analytical methods

Absorption spectra. These were measured in a Unicam spectrophotometer in the range $\lambda = 230$–350 mμ. A line was constructed through the points in the range 350–320 mμ. and extrapolated to shorter wavelengths to give a base line from which the absorption curve corrected for scatter could be calculated (Beaven & Holiday, 1952). The corrected

curve was used to obtain the tyrosine and tryptophan contents using the equations of Goodwin & Morton (1946).

Hydroxyproline was estimated by the method of Neuman & Logan (1950).

Hydrolysis by collagenase. The *Clostridium welchii* collagenase was a gift from Dr C. L. Oakley. It had been prepared by the method of Bidwell & van Heyningen (1948) and was the final product of that fractionation, dried by sublimation after dialysis against borate buffer. It was dissolved in Palitzsch borate buffer pH 7·1 (Clark, 1928) to give a solution containing 15 Q units/ml. (Bidwell & van Heyningen, 1948). The activity of the corneal mucoid and gelatin as substrates for the enzyme was assessed from the amount of non-diffusible N remaining after incubation. Solutions of the mucoid (0·3%) and of gelatin (1·0%) were made in Palitzsch borate buffer, pH 7·1, and 3 ml. samples put in tubes with 1 ml. of the enzyme. The N contents of the mixed solutions and of the enzyme solution were measured and then the mixtures incubated at 37° for 7 hr., 1 ml. withdrawn, dialysed in cellophan against running tap water and the non-diffusible N content measured.

Electrophoresis. This was done in the Tiselius apparatus. The conditions have been described (Woodin, 1952).

Ultracentrifuging. A model E Spinco analytical centrifuge was used. In some cases the balance cell in the rotor was replaced by a 'wedge' cell so that two measurements could be made simultaneously. All measurements were made at 22–25° and at a speed of 59780 rev./min. (250000 *g*).

Osmotic pressures. These were measured in toluene osmometers. Membranes were made from nitrocellulose; a solvent containing 50 ml. ethanol, 50 ml. ether and 7 ml. ethylene glycol gave membranes of suitable permeability. After the osmometers had reached equilibrium at 3–4° the concentration of the mucoid was measured from the N content. When urea was present it was first removed by dialysis.

Other analytical methods were those described by Woodin (1952).

Fractionation of the phenol-insoluble mucoid

Extraction with organic solvents. The dry-matter content of a solution of the phenol-insoluble mucoid was determined and appropriate volumes were dried by sublimation in tubes. Samples were then extracted with formamide, pyridine or ethylene glycol. The solutions were removed and the dry wt. and N content determined after dialysis. The insoluble fraction was dissolved in water and the dry weight and N content were determined after dialysis.

Treatment with trichloroacetic acid and phenol (Aminoff, Morgan & Watkins, 1950). A 0·5% solution of the phenol-insoluble mucoid in water was treated with trichloroacetic acid (final concentration 5%, w/v), left at room temp. for 24 hr., dialysed and freeze-dried. The solid was then extracted with aqueous phenol (92%, w/v). The solution was dialysed and the dry wt. and N content determined. The material remaining insoluble after the phenol treatment was dissolved in water and the dry wt. and N content were determined after dialysis.

Partition between organic solvents and aqueous solution. A solution of the phenol-insoluble mucoid, 0·5% in 0·2M-NaCl (pH 7) was shaken with *n*-butanol and the interfacial precipitate collected. The aqueous phase was shaken again with *n*-butanol and after six extractions removed, dialysed, and the dry wt. and N content were determined. The combined interfacial precipitate was collected, suspended in water, dialysed and the dry wt. and N content were determined. The same procedure was used with the phenol-insoluble mucoid in saturated aqueous $(NH_4)_2SO_4$ with pyridine as the organic solvent and with the phenol-insoluble mucoid in 5M urea, 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 7) with $CHCl_3$–amyl alcohol (4:1, v/v) as the organic solvent.

Treatment with Zeo-Karb 215. A column was set up with 10 ml. wet volume Zeo-Karb 215 (Permutit Co. Ltd., London) in the acid form, and 4 ml. of a solution in water of the phenol-insoluble mucoid were pressed in and washed through with water. The eluate was collected and the hexosamine/N ratio measured.

Fractionation from formamide solution. A solution of the phenol-insoluble mucoid was made in formamide and the non-diffusible N content determined. The solution was treated with ethanol and the precipitates were collected after adding 1·25 and 2·0 vol. ethanol. The precipitates were dissolved in water and the dry wt. and N contents determined after dialysis.

In all cases the dialysis before analysis was against 0·001N-HCl and then distilled water. The volume after dialysis was noted and measured portions were dried in weighed ampoules at 105°. The solid and the ampoule were then incinerated in a Kjeldahl flask and the N content was determined.

RESULTS

Fractionation of the phenol-insoluble mucoid. It was known that the mucopolysaccharide could not be separated from the proteins in the mucoid by shaking with chloroform and amyl alcohol or by extraction with phenol. The chemical fractionations listed in Table 1 also do not dissociate the mucoid. The reagents used would be expected to separate the mucopolysaccharide if its linkage to protein were due solely to hydrogen bonds. Zeo-Karb 215 was used because of the possibility that the interaction was due to the Ca^{2+} used in the extraction of the mucoid. In this case its removal by the resin could enable preferential absorption of the protein on the surface of the resin particles.

The products obtained in these procedures differ in composition by more than the experimental error and this suggests that the mucoid is not a homogeneous substance.

Composition of the mucoid. The mucoid preparations contained 8·3–8·6% N and 20–25% hexosamine. If the non-hexosamine N of the mucoid were protein (16% N), then the mucoid contains 42% protein.

The ultraviolet absorption spectrum of the mucopolysaccharide and of the mucoid are shown in Fig. 1; solutions made in distilled water and in 0·2M-NaCl had identical absorption. The curves for the mucoid at pH 6 and at pH 13, corrected for scatter, show maxima at 275 mμ. and at 290 mμ. respectively, and intersect at 281–282 mμ. Beaven & Holiday (1952) consider an intersection in the

Table 1. *Fractionation of phenol-insoluble mucoid*

The starting material had 8·4–8·6% N and hexosamine/N = 2·6.

Treatment (see text)	Result	Composition of products
Extraction with phenol after trichloroacetic acid treatment	92% recovered in insoluble phase	Insoluble fraction, 8·5% N
Extraction with pyridine	95% recovered in insoluble phase	Insoluble fraction, 8·5% N
Extraction with ethylene glycol	93% recovered in insoluble phase	Insoluble fraction, 8·5% N
Extraction with formamide	80–90% soluble	Insoluble fraction, 8·5% N; soluble fraction, 8·5% N
Solution in 0·2M-NaCl shaken with *n*-butanol	After 6 extractions 30% recovered in precipitate, 60% recovered in solution	Soluble fraction, 8·8% N; precipitate, 7·9% N
Solution in sat. $(NH_4)_2SO_4$ shaken with pyridine	After 4 extractions 20% recovered in precipitate, 72% recovered in solution	Precipitate 8·7% N; soluble fraction 8·1% N
Solution in 5M urea shaken with chloroform and amyl alcohol	After 6 extractions 34% in precipitate, 54% in solution	Precipitate, 8·9% N; soluble fraction, 8·2% N
Solution in formamide treated with ethanol	75% precipitated by 1·25 vol. ethanol; 15% precipitated on increasing ethanol to 2 vol.	First precipitate 8·8% N, 2nd precipitate, 7·9% N
Solution in distilled water passed down column of Zeo-Karb 215	80% recovered in eluate	Eluate had hexosamine/N = 2·8

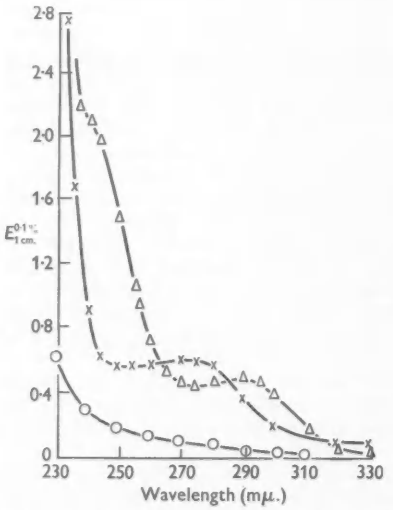

Fig. 1. Ultraviolet absorption spectra of the mucoid and mucopolysaccharide: ×—×, phenol-insoluble mucoid in distilled water; △—△, phenol-insoluble mucoid in 0·1N-NaOH; ○—○, mucopolysaccharide in distilled water.

range 276–281 mμ. to be evidence that only tyrosine and tryptophan are contributing significantly to the absorption in the region. The tyrosine content of the mucoid on this assumption is 2·4–2·5% and the tryptophan content 0·8–0·9%. If the mucoid contains 40% protein, then that protein contains 6% tyrosine and 2% tryptophan. The absorption curve of the gelatin analysed in the same way gave, tyrosine 0·8%, tryptophan 0·25%. It is not claimed that these figures are those for pure corneal collagen, but it is clear that the mucoid protein contains more tyrosine and tryptophan than does the collagen.

The hydroxyproline content of the mucoid was 0·5%, of the gelatin 10%.

After 7 hr. incubation with collagenase 90% of the corneal gelatin, but less than 15% of the mucoid diffused through the dialysis membrane.

Electrophoresis. If the protein and the mucopolysaccharide are combined by a specific interaction of their charged groups, the anionic groups of the polysaccharide combining with the cationic groups of the protein, then at a pH where the ionization of the latter is suppressed dissociation should occur. The p*K* for the second stage of the dissociation of sulphuric acid is given by Glasstone (1942) as 1·02. It is to be expected, therefore, that the ionization of the sulphuric ester groups of the mucopolysaccharide will be complete above pH 3. The effect of increasing the pH above this value will be to induce the ionization of the carboxyl groups and then, successively, to suppress the ionization of the imino, α-amino, ε-amino, and guanidino groups. This will have the effect of increasing the overall repulsion between the protein and the polysaccharide and will also tend to abolish any specific interaction between the cationic groups of the protein and the sulphuric ester groups.

Fig. 2 shows the pH/mobility curve for the phenol-insoluble mucoid and Fig. 3 the schlieren diagrams for the phenol-insoluble mucoid migrating under various conditions. Below pH 4 the solutions became increasingly opalescent and for measurements below pH 2 it was necessary to use a mucoid concentration of 0·2%. Electrophoresis at a pH where ionization of the sulphuric ester groups would be suppressed was considered impracticable because of this and of the high conductivity of the solution.

The phenol-insoluble mucoid migrated with a single boundary in the range pH 2–11·5 in buffers containing 0·18M-NaCl or in 0·5M-NaCl (pH 6·7). In the presence of guanidine hydrochloride (0·5M) at pH 6·6 the peak becomes asymmetric and the mobility is reduced to about half the value it has in 0·5M-NaCl. These two solvents have almost the same conductivity. At pH 12 the peaks in the two sides of the cell are no longer identical, the σ boundary is larger than at lower pH and there is partial resolution into two components. There is also evidence of dissociation at pH 1·4, but because of the low concentration of mucoid and the opalescence of the solutions, this is less evident than at pH 12. Moreover, at pH 1·4 the mucopolysaccharide and protein would probably be oppositely charged and their interaction different from that at a higher pH.

Samples of the residues from the osmotic-pressure measurements made at pH 12 and also in 5M urea (pH 7) were dialysed against NaCl ($I=0·18$), sodium phosphate buffer ($I=0·02$, pH 6). On electrophoresis a single symmetrical boundary was observed.

Osmotic pressure. A straight line is not obtained by plotting P/C (P = osmotic pressure in mm. Hg, C = concn. as %, w/v) against concentration. The plot of $\log_{10} P/C$ against concentration gives a better approximation to a straight line and this method was used to obtain, by extrapolation, the values of P/C at infinite dilution. Because of the heterogeneity of the mucoid these values are useful only as a measure of the number of particles in unit weight of mucoid.

Fig. 4 gives the osmotic-pressure data for a sample of the mucoid precipitated from formamide in salt solution, in alkali and in urea and also for the residues from the urea and alkali returned to the

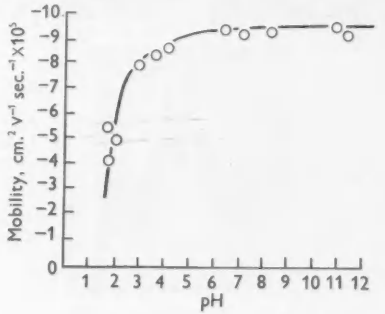

Fig. 2. pH/mobility curve for the phenol-insoluble mucoid. The mobilities are the means of the values for the boundaries in both sides of the cell. The solvents were:

pH	Composition of solvent		
1·48	0·16M-NaCl	0·04M-HCl	—
1·60	0·17M-NaCl	0·03M-HCl	—
1·93	0·186M-NaCl	0·0142M-HCl	—
2·62	0·18M-NaCl	0·02M-HCl	0·04M glycine
3·8	0·18M-NaCl	0·02M sodium acetate	0·13M acetic acid
4·1	0·18M-NaCl	0·02M sodium acetate	0·07M acetic acid
6·1	0·18M-NaCl	0·0027M-Na_2HPO_4	0·0122M-NaH_2PO_4
7·1	0·18M-NaCl	0·0057M-Na_2HPO_4	0·0029M-NaH_2PO_4
8·25	0·18M-NaCl	0·0066M-Na_2HPO_4	0·0006M-NaH_2PO_4
10·65	0·18M-NaCl	0·007M-Na_2CO_3	—
11·4	0·18M-NaCl	0·0017M-Na_2HPO_4	0·0026M-Na_3PO_4

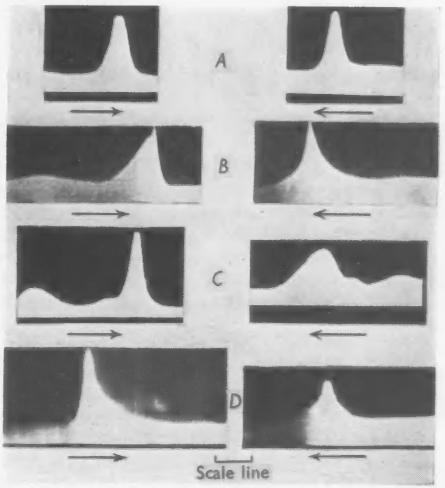

Fig. 3. Schlieren diagrams for the phenol-insoluble mucoid migrating on electrophoresis. The ascending boundary is on the left-hand side and the arrows indicate the direction of migration which in all cases was towards the anode. The scale line represents 1 cm. in the electrophoresis cell for all diagrams. The light source was a mercury vapour lamp with a filter giving light at 546 mμ. *A*: mucoid in NaCl (0·5M), NaH_2PO_4 (0·0029M), Na_2HPO_4 (0·0057M), pH 6·75, after 400 min. at 0·8v/cm. *B*: mucoid in guanidine hydrochloride (0·5M), NaH_2PO_4 (0·0029M), Na_2HPO_4 (0·0057M), pH 6·6, after 1100 min. at 0·85v/cm. *C*: mucoid in NaCl (0·18M), NaOH (0·02M), pH 12·1, after 200 min. at 1·7v/cm. *D*: mucoid in NaCl (0·17M), HCl (0·03M), pH 1·58, after 90 min. at 1·17v/cm. and 180 min. at 1·95v/cm.

salt solution. Table 2 gives the range of values obtained with different samples of mucoid. The mucoid is dissociated by urea and at pH 12; these effects are partly reversible.

Ultracentrifuge data. Fig. 5 shows the schlieren diagrams of the mucoid precipitated from formamide and the mucopolysaccharide prepared from this material, sedimenting in various solvents. Base lines were constructed to these diagrams and the peaks reduced to this as origin. The constructed curves are shown in Fig. 6. Two samples of the mucoid precpitated from formamide and one sample of the phenol-insoluble mucoid have given essentially the same results.

In 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 7), and in 2·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 7), the mucoid has three components but in 5 M urea, 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 7), the mucoid sediments with a single asymmetric boundary. After standing in 5 M urea solution for several days followed by dialysis against 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 7), the mucoid shows three components but the contribution of the fastest to the total refraction is smaller than in the mucoid before treatment with urea.

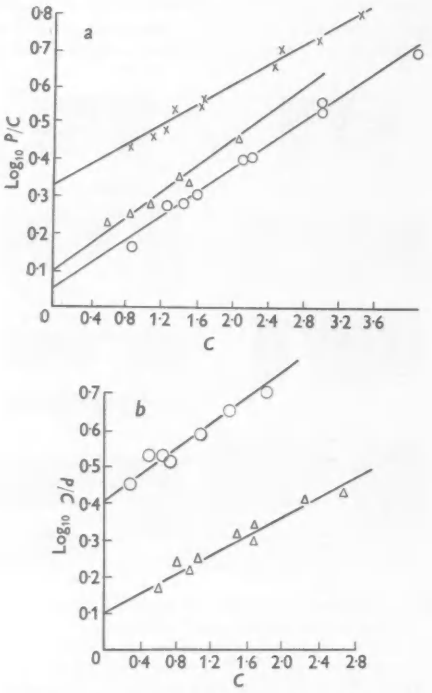

Fig. 4. Osmotic pressure of the phenol-insoluble mucoid. P = Pressure in mm. Hg; C = concentration as g. mucoid/100 ml. solution. (*a*) ○—○, mucoid in 0·5 M-NaCl, 0·05 M sodium phosphate buffer (pH 7); ×—×, mucoid in 0·5 M-KCl, 0·02 M-NaOH (pH 12·1); △—△, mucoid, after exposure to pH 12·1, in 0·5 M-NaCl, 0·05 M sodium phosphate buffer (pH 7). (*b*) ○—○, mucoid in 5 M urea, 0·5 M-NaCl, 0·05 M sodium phosphate buffer (pH 7); △—△, mucoid, after exposure to 5 M urea, in 0·5 M-NaCl, 0·05 M sodium phosphate buffer (pH 7).

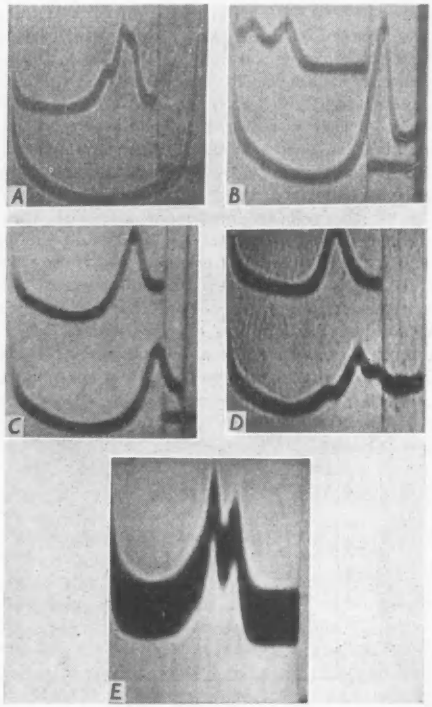

Fig. 5. Schlieren diagrams of the mucoid precipitated from formamide and mucopolysaccharide sedimenting on ultracentrifuging. *A* and *B* upper diagram: mucoid in 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 8). Lower diagram: mucoid in 5 M urea, 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 8). *A*, 70 min. after reaching full speed. *B*, 240 min. after reaching full speed. *C*, upper diagram: mixture of mucopolysaccharide and mucoid in 5 M urea, 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 8). Lower diagram: mucopolysaccharide in 5 M urea, 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 8), 163 min. after reaching full speed. *D*, upper diagram: mucopolysaccharide in 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 8). Lower diagram: mucoid in 2·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 8), 161 min. after reaching full speed. *E*, residues from urea treatment: 0·2 M-NaCl, 0·05 M sodium phosphate buffer (pH 7), 170 min. after reaching full speed.

Table 2. *Osmotic-pressure data on mucoid preparations*

'Phosphate' signifies sodium phosphate buffer. P = pressure in mm. Hg; C = concn. as % w/v.

Solute	Solvent	pH	P/C ($C \rightarrow 0$)
Mucoid precipitated from formamide	NaCl 0·5M Phosphate 0·05M	7	0·9–1·05
Mucoid precipitated from formamide	NaCl 0·5M Na_2CO_3 0·05M	10·5	0·9
Mucoid precipitated from formamide	Urea 5M NaCl 0·5M Phosphate 0·05M	7	2·5–2·8
Mucoid precipitated from formamide	KCl 0·5M NaOH 0·02M	12·1	2·0–2·15
Mucoid precipitated from formamide (after NaOH treatment)	NaCl 0·5M Phosphate 0·05M	7	1·2
Mucoid precipitated from formamide (after urea treatment)	NaCl 0·5M Phosphate 0·05M	7	1·2
Phenol-insoluble mucoid	NaCl 0·5M Phosphate 0·05M	7	0·95
Phenol-insoluble mucoid	Urea 5M NaCl 0·5M Phosphate 0·05M	7	2·95
Phenol-insoluble mucoid (after urea treatment)	NaCl 0·5M Phosphate 0·05M	7	1·15

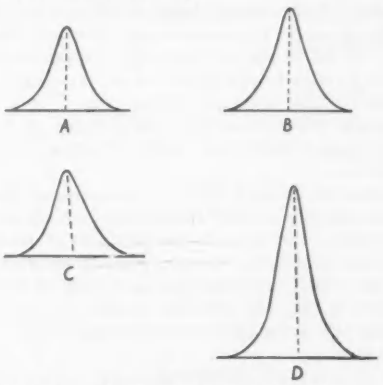

Fig. 6. Schlieren diagrams of the mucoid and mucopolysaccharide sedimenting in the ultracentrifuge. The curves are corrected for the variation in the refractive index of the solvent in the direction of sedimentation. *A*, mucopolysaccharide in 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 8); *B*, mucopolysaccharide in 5M urea, 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 8); *C*, mixture of mucoid and mucopolysaccharide in 5M urea, 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 8); *D*, mucoid in 5M urea, 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 8). The broken lines are drawn through the midpoints of the bases of the diagrams and their departure from the vertical is a measure of the asymmetry.

The mucopolysaccharide sediments with a single boundary in both 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 7), and in 5M urea, 0·2M-NaCl, 0·05M sodium phosphate buffer (pH 7). A mixture of the mucopolysaccharide and the mucoid in urea solution sediments with a single boundary.

DISCUSSION

During sedimentation in the centrifugal field the mucoid in salt solution separates into three components but the mucopolysaccharide under the same conditions moves with a single boundary. The heterogeneity of the mucoid could be due to combination of the mucopolysaccharide with proteins of different weight or shape or to polymerization of different proportions of mucopolysaccharide and protein particles. The fact that the dissociation of the fastest-sedimenting component by urea is not completely reversible suggests that it is such a polymer.

The tyrosine, tryptophan and hydroxyproline contents, and the reaction with collagenase, indicate that the mucoid contains proteins other than collagen derivatives. The heterogeneity of the mucoid makes impossible the rigorous exclusion of the presence of collagen, but the suggestion made previously, that the mucoid protein is derived from corneal collagen can no longer be accepted (Woodin, 1952).

The mucoid migrates in the electrical field with a single boundary in the range pH 2–11·5. At a higher pH there is partial resolution into two components but the sharp appearance of the ascending boundary and the incomplete resolution of the descending boundary suggest rather, that the mucoid is migrating as an equilibrium mixture with its dissociation products. If the values of the dissociation constants for the α-amino, carboxyl, imino, and lysine ε-amino groups, given by Cohn & Edsall (1943), are those which apply to these groups combined in the mucoid, then their participation in

a specific electrostatic linkage can be excluded on account of the single boundary observed on electrophoresis. However, in absence of knowledge of the effect of the negative charge of the mucopolysaccharide on these dissociation constants, it is possible that the ε-amino group of lysine still has a positive charge at pH 11·5 and the dissociation observed at pH 12 could be due to suppression of that charge. It is clear, from the relative contribution of the two components at pH 12 to the total refraction, that the slower cannot account for all the protein combined in the mucoid. That protein still combined above pH 12, if it is combined electrostatically, must be so by virtue of the charge on the amidine group of the arginine and a more probable explanation of the dissociation at pH 12 is that it is due to partial suppression of the ionization of these groups.

In guanidine hydrochloride solutions the mucoid migrates with a markedly asymmetric boundary. This condition could arise if the mucoid were migrating as an equilibrium mixture with its dissociation products. There is also a reduction in the mobility of the mucoid in this solvent, a condition which is taken as evidence for a strong interaction of the guanidine and the sulphuric ester groups.

Implicit in this analysis of the electrophoretic behaviour is the assumption that the protein and mucopolysaccharide will differ sufficiently in their net charge to permit separation where dissociation occurs. This is likely, as the mucoid has a normal distribution of amino acids (Woodin, 1952) and the protein will have normal amphoteric properties.

The osmotic-pressure measurements provide confirmation of the reversible dissociation of the mucoid by alkali or the amidinium ion. In the presence of urea or at pH 12, but not at pH 10·5, there is an increase in (number of particles/unit weight of mucoid) over the value observed at pH 7 in salt solutions. If the action of urea and alkali is, as is suggested here, to set up an equilibrium between the mucoid and its products of dissociation, then further dissociation should be favoured by dilution and that would reveal itself by values of P/C which increase with dilution. The deviation of the mucoid under all conditions from ideal behaviour is so great, that it cannot be said if this occurs, but the fact that $\log_{10} P/C$ is a linear function of concentration may be of significance.

The single boundary observed when the mucoid sediments in urea solution could arise if the products of dissociation have identical sedimentation rates or if there were set up an equilibrium of the mucoid and its dissociation products. The boundary is asymmetric but there is no tendency to separation into separate peaks, suggesting a continuous distribution of sedimentation rates. This could arise if there were, in urea solution, an equilibrium of the type:

$$AB \rightleftharpoons A+B, \quad AC \rightleftharpoons A+C, \quad AD \rightleftharpoons A+D,$$

combination of A with B, C, or D being an equally probable event. The single boundary observed in the mixture of the mucopolysaccharide and mucoid in urea could then arise if the added mucopolysaccharide participated in that equilibrium.

It is possible that a further analysis of the action of urea and guanidine will permit identification of the linkage between the mucopolysaccharide and protein in the mucoid. The electrophoretic behaviour indicates that an electrostatic linkage, if present, is most probably between the sulphuric ester groups and the arginine side chains. If there were present in the mucoid such a sulphuric ester–arginine linkage, then urea and guanidine might induce dissociation by competing with the arginine for the sulphuric ester groups. That the guanidinium cation could have such an action can be readily understood (Walker, 1949). Harris & Robson (1948) have suggested that the dipolar form of urea should be regarded as a strongly basic substituted amidine, but evidence for the interaction of urea and resonating anions of the sulphuric ester type does not appear to have been sought. An action of urea and guanidine analogous to that suggested here was originally put forward by Tsao, Bailey & Adair (1951) to account for the depolymerization of tropomyosin.

That urea and guanidine do have this action on the corneal mucoid must remain a hypothesis till it is possible to establish that the products of dissociation are indeed the mucopolysaccharide and the protein. The only evidence in favour of this at present is that the mixture of the mucopolysaccharide and dissociated mucoid sediments as a single component.

SUMMARY

1. The tyrosine, tryptophan and hydroxyproline contents and the reaction with collagenase indicate that corneal mucoid contains proteins which are not collagen derivatives.

2. The mucoid migrates with a single boundary on electrophoresis in the range pH 2–11·5. At higher pH, or in presence of guanidine, the peak is asymmetric and the mucoid behaves as an equilibrium mixture with its dissociation products.

3. Osmotic pressure measurements reveal that the mucoid is dissociated by urea or at pH 12. These effects are partly reversible.

4. The derived mucopolysaccharide sediments with a single boundary in the ultracentrifuge. The mucoid has three components in salt solutions, but in urea solution it moves with a single boundary. A mixture of the mucoid and mucopolysaccharide in urea solution sediments with a single boundary.

5. The relevance of these observations to the problem of the protein–mucopolysaccharide linkage is discussed and the hypothesis put forward that it is due to ion-pair formation between sulphuric ester groups and the amidine groups of arginine residues.

I wish to thank Dr A. S. McFarlane for his hospitality at the National Institute for Medical Research and Dr T.-C. Tsao for many invaluable suggestions. The preliminary ultracentrifuge work was done at the Massachusetts General Hospital, Boston, and I am very grateful to Dr Karl Schmid and Dr Jerome Gross for making this possible and for the use of the machine. I am also indebted to Dr C. L. Oakley for the gift of the collagenase preparation.

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The Isolation of *allo*Hydroxy-L-Proline from Sandal (*Santalum album* L.)

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During the course of investigations on the nitrogen metabolism of plants by the radial ('circular') paper-chromatographic technique, the presence of considerable quantities of free proline in leaves of several plant species and hydroxyproline in the leaves of sandal was observed (Giri, Gopalkrishnan, Radhakrishnan & Vaidyanathan, 1952). Examination of paper chromatograms of an ethanolic extract of the leaves of *Santalum album* showed that the band corresponding to hydroxyproline was quite prominent and an attempt was therefore made to isolate the substance in a pure state. In the present paper an account is given of its isolation and identification as *allo*hydroxy-L-proline.

The procedure of Town (1928) was not found suitable in this case. However, a partial purification was initially effected by absorption over a column of Zeo-Karb H.I., whereby some neutral constituents and a few of the amino acids were removed. By chromatography of the eluates on cellulose powder and copper carbonate–alumina columns, the purification of the substance was sufficient to induce crystallization. Failure to isolate the amino acid by Town's procedure is believed to be due to the presence of an accompanying substance which yields a picrate (m.p. 335°) but is otherwise as yet unidentified.

The purified amino acid gave correct analytical values and melted at 248° (decomp.). The melting point as well as the optical rotation are in good agreement with values previously reported for *allo*hydroxy-L-proline. A mixed melting point with an authentic sample was unchanged. Further, both the samples were indistinguishable in their behaviour on paper chromatograms and also crystallized in the same characteristic broom-like manner on a microscope slide. Additional evidence as to their identity was provided by X-ray powder photographs.

EXPERIMENTAL

Identification of free amino acids in leaves of Santalum album

Extraction. Fresh leaves were dried in an oven at 50° and powdered (200 mesh/in.). A sample (0·2 g.) of the powder was extracted for 1 hr. in the cold with 75% (v/v) aqueous ethanol (2 ml.). After centrifuging, the supernatant was shaken with $CHCl_3$ (3 vol.). The aqueous layer containing all the amino acids was separated by centrifuging and examined by paper chromatography. Clear separation of the bands could be achieved with 10–20 μl. of the aqueous extract.

Paper chromatography. Whatman no. 1 filter-paper disks (25 cm. diam.) were used and the chromatograms developed

with *n*-butanol–acetic acid–water solvent (4:1:5 by vol.), as described by Giri & Rao (1952*a*, *b*). The dried papers, on spraying with 0·1% (w/v) ninhydrin in 95% (v/v) aqueous acetone and heating at 60° (30 min.), showed bands (Fig.1) corresponding to the following amino acids: leucine + isoleucine, phenylalanine, valine + methionine, tyrosine, proline, alanine, glutamic acid, hydroxyproline, arginine, histidine, lysine + ornithine and an unidentified peptide. The characteristic yellow bands of proline and hydroxyproline were prominent.

Confirmatory tests. Specific tests for hydroxyproline were carried out on a radial chromatogram of the aqueous extract by the 'multiple sector' technique. While with the isatin reagent (Acher, Fromageot & Jutisz, 1950) proline gave a dark-blue colour, the band corresponding to hydroxyproline gave a lighter duck-egg blue. With Folin's reagent (Giri & Nagabhusanam, 1952) the hydroxyproline gave an orange band. The combined reagents, alloxan–isatin (Harris & Pollock, 1953) and isatin–*p*-dimethylaminobenzaldehyde also gave a positive test. The latter reagent constitutes a highly sensitive and specific test for hydroxyproline (Jepson & Smith, 1953).

Estimation of hydroxyproline by the method of Neuman & Logan (1950) gave 1 mg./ml. of the aqueous extract employed for chromatography and this agreed well with the value (0·99 mg./ml.) obtained after separation of the amino acid by radial paper chromatography as follows. Chromatograms with 10 μl. of the extract applied at four points near the centre of a Whatman no. 1 paper disk (25 cm. diam.) were developed with the butanol–acetic acid–water mixture. After drying, the opposite segments were sprayed with ninhydrin to serve as guide strips for the remaining pair, from which the bands corresponding to hydroxyproline were cut out, eluted with water and estimated as before.

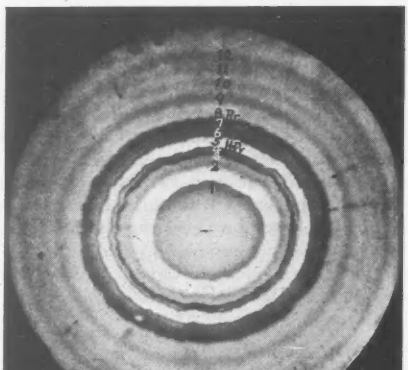

Fig. 1. Radial paper chromatogram of amino acids in ethanol extract of leaves of *Santalum album*, showing the position of *allo*hydroxy-L-proline. 1, Unidentified peptide; 2, lysine + ornithine; 3, histidine; 4, arginine; 5, hydroxyproline; 6, glutamic acid; 7, alanine; 8, proline; 9, tyrosine; 10, valine + methionine; 11, phenylalanine; 12, leucine + isoleucine. (For conditions see text.)

Isolation of allo*hydroxy-*L*-proline from leaves of* Santalum album

Material. Freshly collected sandal leaves free from twigs were cut, dried at 50° for 48 hr., and powdered in a mortar to 200 mesh/in.

Extraction. The powder (100 g.) was extracted for 2 hr. with hot 75% (v/v) aqueous ethanol (1 l.) and filtered. The residue was again extracted with the solvent (500 ml.). The combined extracts were evaporated to dryness *in vacuo*, taken up with water (20 ml.) and filtered to remove chlorophyll.

Purification over Zeo-Karb H.I. The dark-brown extract was placed at the top of a prepared column (2·5 × 50 cm.) of Zeo-Karb H.I. (Permutit Co. Ltd., London) and developed with water (200 ml.), when most of the amino acids (including hydroxyproline, the colouring matter and neutral constituents) were found in the eluate. On displacing the column by 10% (w/v) HCl, about 50% of the original solids was obtained as a partly crystalline mixture (containing only a small percentage of hydroxyproline) which was not further examined.

Fractionation of the amino acids by displacement chromatography. The aqueous eluate, which had a high concentration of hydroxyproline, was evaporated *in vacuo* to 20 ml. and fractionated on a second column of Zeo-Karb H.I. (2·5 × 50 cm.). On development with water, all the amino acids were absorbed leaving neutral materials and colouring matter in the eluate. The amino acids were displaced by 10% (w/v) aqueous HCl (300 ml.) and the effluent collected in 10 ml. fractions. The progress of fractionation of the amino acid was followed by radial paper chromatography. Hydroxyproline was present in the fractions 6–21, mixed with small quantities of other amino acids. The fractions 8–15, containing histidine and arginine also, were pooled and purified further by paper chromatography. The remaining fractions were used for the cellulose powder and copper carbonate–alumina columns.

Isolation by paper chromatography. A sample from the fractions 8–15, containing about 8–10 mg. of hydroxyproline was applied at the centre of a Whatman no. 1 (or, better, no. 3) disk (35 cm. diam.) and developed with the *n*-butanol–acetic acid–water solvent. Good resolution was attained by repeated drying and development with fresh solvent. As before, guide strips of 0·5 cm. width sprayed with ninhydrin were used to indicate the position of the amino acid. The bands were cut out from the chromatograms, packed in a small column and extracted with 75% (v/v) aqueous ethanol. Evaporation of the solvent *in vacuo* gave a residue containing most of the hydroxyproline with only traces of histidine and arginine. Although it was chromatographically homogeneous, the residue gave low values for hydroxyproline as estimated by the method of Neuman & Logan. However, on treatment with glacial acetic acid or aqueous ammonia (sp.gr. 0·880), a ninhydrin-negative material, not resolved chromatographically from hydroxyproline, was left undissolved. Its chemical nature is still unknown. The acetic acid extract was evaporated *in vacuo* to dryness, the residue dissolved in the minimum quantity of water, decolorized with charcoal and precipitated by addition of absolute ethanol. By a repetition of this process the amino acid was obtained in a pure state.

Fractionation on a column of copper carbonate–alumina. The hydroxyproline-rich fractions 6, 7 and 16–21 suitably

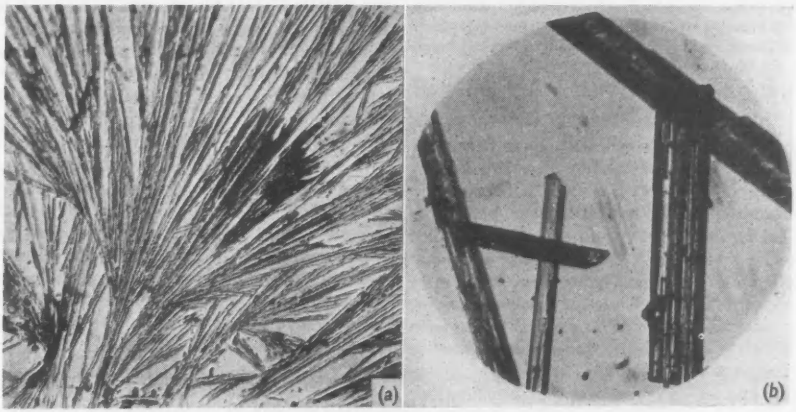

Fig. 2. Photomicrographs of crystals of natural *allo*hydroxy-L-proline: (*a*) as crystallized from aqueous ethanol on microscope slide; (*b*) as crystallized from *n*-propanol on slide (× 120).

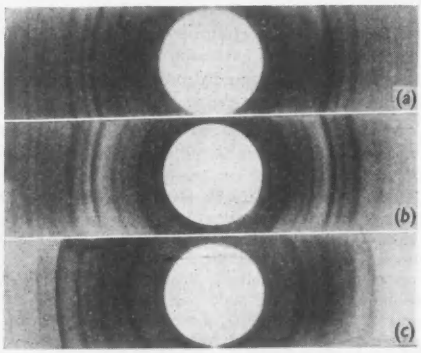

Fig. 3. X-ray powder photographs (paper positive) of hydroxyproline specimens (Cr radiation, V filter; camera diameter 5·7 cm.). (*a*) Authentic sample of *allo*-hydroxy-L-proline; (*b*) natural *allo*hydroxy-L-proline as obtained from *S. album*; (*c*) hydroxyproline.

diluted were passed through a column of basic copper carbonate–alumina (1:3) according to the method of Thompson, Pollard & Steward (1953). The basic copper carbonate used by us was a mixture of native malachite and azurite and the column was packed dry. The eluates were collected in 10 ml. fractions. Fractions 12–21, containing hydroxyproline, were freed from Cu^{2+} by passing H_2S. The residue obtained on evaporation *in vacuo* was purified as described before with glacial acetic acid.

Fractionation on a column of cellulose powder. Fractions 5–11 and 22–26 from the copper carbonate–alumina column containing mostly hydroxyproline were purified on a column of cellulose powder prepared according to Synge (1951). The solvent mixture, ethanol–acetic acid–water (90:2:8 by vol.), was employed both for preparing the column and for development. The effluent was collected in 10 ml. fractions. Hydroxyproline from fractions 10–24 was purified as before.

Crystallization. It was difficult to induce crystallization from aqueous ethanol unless the impurities were removed by treatment with glacial acetic acid or aqueous ammonia (sp.gr. 0·880). However, it was found that *allo*hydroxy-L-proline crystallizes best from aqueous *n*-propanol. By slow evaporation from this solvent at 50° it separated in thin, long, glistening needles. By all the methods employed above a total of approx. 100 mg. of the pure imino acid was obtained.

Identification of the natural amino acid as allo*hydroxy-L-proline.* The natural material was compared with synthetic *allo*hydroxy-L-proline (Robinson & Greenstein, 1952). The isolated amino acid was highly soluble in water and aqueous ethanol but practically insoluble in absolute ethanol and *n*-propanol. Both the natural and the synthetic samples of *allo*hydroxy-L-proline crystallized alike from aqueous *n*-propanol and Fig. 2 (*b*) shows a photomicrograph of the crystals. In Fig. 2 (*a*) can be seen the characteristic broom-like manner in which it crystallizes on a microscope slide from water or ethanol.

M.p. was 248° (uncorr., decomp.). When admixed with an authentic sample of *allo*hydroxy-L-proline (m.p. 246–247°) m.p. was 247°. Specific optical rotation (0·5 dm. microtube) was $[\alpha]_D - 57°$ (water, $c = 0{\cdot}65$). Neuberger (1945) reported $[\alpha]_D - 58°$, m.p. 248°, and Wieland & Witkop (1940) $[\alpha]_D - 57{\cdot}4°$, m.p. 241°.

Elementary analysis (Department of Organic Chemistry of this Institute) gave the following values. (Found: C, 45·6; H, 6·7; N, 10·5. Calc. for $C_5H_9O_3N$: C, 45·7; H, 6·9; N, 10·7%.)

Chromatographic behaviour. The isolated imino acid could not be resolved from *allo*hydroxy-L-proline on a chromatogram with the butanol–acetic acid–water solvent, nor with pyridine–water (4:1, v/v), phenol–water or ethanol–acetic acid–water (90:2:8 by vol.). Its behaviour with colour reagents was also identical with that of the authentic sample.

X-ray diffraction. The X-ray powder photographs of *allo*-hydroxy-L-proline (*a*) and the isolated compound (*b*), which are identical, are given in Fig. 3; that of hydroxy-L-proline (*c*) is given for the sake of reference.*

DISCUSSION

The experimental results obtained have furnished unequivocal evidence for the identity of the compound with *allo*hydroxy-L-proline. Using only paper-chromatographic identification, this was reported earlier as hydroxyproline (Giri *et al.* 1952). Thus for the first time free *allo*hydroxy-L-proline has been detected in plants, being now obtained in crystalline form from the leaves of *Santalum album*. The only previous report of its natural occurrence was by Wieland & Witkop (1940), who isolated it from hydrolysates of phalloidin.

Using only paper-chromatographic identification, hydroxyproline has further been reported to occur free in pollen (Auclair & Jamieson, 1948), in prunes (Joslyn & Stepka, 1949), in the haemolymph of *Drosophila melanogaster* (Auclair & Dubreuil, 1953), in the sporulation medium of *Bacillus globigii* (Davis & Williams, 1952) and in the blood and Malpighian tubes of the larvae of *Bombyx mori* infected with polyhedral disease (Drilhon, Busnel & Vago, 1951). Besides its well-known occurrence in collagens and gelatins, it has been reported in a combined state in alfalfa protein (Steward, Thompson & Millar, 1951), in sugar-beet protein (Sisakyan, Bezinger & Kuvaeva, 1951; Vavruch, 1952), in *Sarcina lutea* (Belozerskiĭ & Kireenkova, 1943), dentine protein (Losee, Neidig & Hess, 1951), proteins of the insect cuticle (Hackman, 1953) and horseradish peroxidase (Machly & Paleùs, 1950). Hydroxyprolines are not, however, found free or combined in most biological materials that have been examined by paper chromatography. Moreover, according to Steward & Thompson (1950), hydroxyproline has never been found free in plants. Chromatographically the isomers have not been differentiated. Since it is now known that besides these isomers, many other compounds behave somewhat similarly on paper chromatograms, previous claims regarding the presence of hydroxyproline in natural sources obviously require further substantiation, preferably by isolation.

*allo*Hydroxy-L-proline has been found to be present in sandal tree largely in the free state. It appears to be more concentrated in the flowers and the pericarp of the fruit than in the leaves. The proportion of hydroxyproline N to total N in samples of flowers and pericarp was found to be 31 and 45% respectively (Radhakrishnan, Gopalkrishnan & Giri, unpublished). Its presence in these materials may be related to its progressive movement from the leaves. In view of the occurrence of a high percentage of *allo*hydroxy-L-proline in the free state, *S. album* might be an economic source for its production on a large scale.

The role played by this amino acid in plant physiology is not known. A knowledge of the changes in the hydroxyproline content in normal and pathological (spiked) conditions of the tree will be of considerable physiological interest and work on this subject is in progress.

* Infrared spectra of *allo*hydroxy-L-proline and the natural preparation were studied by the kindness of Professor R. S. Krishnan, of the Department of Physics of this Institute, with a Perkin-Elmer single-beam automatic recording instrument, model 112, with rock-salt optics. The spectra were essentially identical in the region investigated (2–15μ.).

SUMMARY

1. The natural occurrence of free *allo*hydroxy-L-proline is reported for the first time. A detailed account is given of its isolation from the leaves of sandal (*Santalum album*).

2. The substance was isolated in crystalline form by radial ('circular') paper chromatography, chromatography on columns of cellulose powder and of basic copper carbonate–alumina, following a previous partial separation from other amino acids by ion-exchange chromatography.

3. The isolated amino acid has been identified as *allo*hydroxy-L-proline by numerous chemical and physical criteria.

Our thanks are due to Professor Jesse P. Greenstein (National Institute of Health, Bethesda) for a gift of the isomers of hydroxyproline and to Professor F. C. Steward (Cornell University) and Dr John F. Thompson (U.S. Department of Agriculture, Ithaca) for their interest throughout this work and, particularly, for supplying details of the $CuCO_3$–Al_2O_3 procedure before publication. We are indebted to Dr P. L. Narasimha Rao of this Department for helpful discussion. Our thanks are also due to Dr E. G. Ramachandran (Department of Metallurgy) for the X-ray photographs. One of us (A. N. R.) was a Government of India Senior Scholar during this work.

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Mode of Action of Vitamin D. The Effect of Vitamin D Deficiency in the Rat on Anaerobic Glycolysis and Pyruvate Oxidation by Epiphyseal Cartilage

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The role of vitamin D in calcification of bone still awaits elucidation; this is not to be wondered at since the mechanism of normal calcification is still far from clearly understood. The demonstration of alkaline phosphatase in bone and hypertrophic cartilage by Robison (1923) and Robison & Soames (1924) led to the suggestion that the enzyme promoted calcification by causing a local increase in concentration of inorganic phosphate. That this hypothesis did not explain fully the process of calcification was recognized by Robison, who postulated the presence of a second mechanism which was susceptible to the action of cyanide, fluoride and iodoacetate (Robison, MacLeod & Rosenheim, 1930; Robison & Rosenheim, 1934). Though Robison & Rosenheim (1934) did not make any suggestion about the nature of this second mechanism, in the light of the later knowledge of the action of the above-mentioned inhibitors in the intermediate metabolism of carbohydrates, it may be permissible to conclude that Robison's second mechanism must be very intimately connected with glycogen metabolism in hypertrophic cartilage.

Gutman & Gutman (1941) provided experimental evidence associating glycolysis with calcification occurring in calcifying cartilage. They demonstrated the presence of phosphorylase in significant concentration in the epiphyses of growing rats and rabbits and suggested that this enzyme could initiate conversion of the glycogen of hypertrophic cartilage cells into phosphoric ester substrates for cartilage phosphatase. Later Gutman, Warrick & Gutman (1942) observed that the addition of 0·0005 M phloretin, which is a specific inhibitor for phosphorylase, completely blocked the calcification of rachitic cartilage in solutions containing calcium and inorganic phosphorus in concentrations of 8 and 5 mg. respectively/100 ml. of the medium. The addition of glucose 1-phosphate (G 1-P) restored the capacity of the cartilage to calcify even in the presence of phloretin. The authors concluded, therefore, that phosphorylase was necessary in the calcification mechanism. Further work with the use of inhibitors like cyanide, iodoacetate and fluoride suggested that the calcification of cartilage involved phosphorylative glycogenolysis at least to the point of phosphopyruvate formation.

Additional evidence in support of the important role of glycogenolysis in initiating calcification was forthcoming from the work of Albaum, Hirschfeld & Sobel (1952*a*) who observed that adenosine triphosphate (ATP), which is essential in these enzymic processes, was present in epiphyseal cartilage of rat tibiae at the same level as was normally found in liver and kidney (9·2–17·2 μg./rat, i.e. two tibiae). Albaum, Hirschfeld & Sobel (1952*b*) further demonstrated the presence of most of the glycogenolytic enzymes in epiphyseal cartilage and several of the phosphorylated intermediates by a chromatographic technique.

Although the role of glycogenolysis in calcification had thus been clearly demonstrated, current opinion appears to view this process only from the standpoint of its being able to provide phosphoric esters, which under the influence of alkaline phosphatase would provide the requisite concentration of inorganic phosphorus for the deposition of the bone salt. This concept, which lays emphasis on the

concentrations of calcium and inorganic phosphorus alone, does not fully explain the observed facts.

Dikshit & Patwardhan (1952) have shown that the epiphyseal cartilage of rachitic rat after treatment with vitamin D can undergo calcification *in vitro* in media in which the cartilage of untreated rats fails to calcify. These authors suggested that, on administration of vitamin D, some changes as yet ill understood take place in the cartilage which impart to the latter the capacity to calcify. A histochemical examination of rachitic epiphyseal cartilage from rats before and after treatment with vitamin D has recently been made by Ramalingaswami, Sriramachari, Dikshit, Tulpule & Patwardhan (1954). As a result of these studies, the authors concluded that failure of calcification in vitamin D deficiency probably was due to a disturbance or arrest of the metabolism of glycogen in the rachitic cartilage. Experimental evidence that such disturbance does in fact take place in induced rickets in rats is described in the present communication.

EXPERIMENTAL

The anaerobic breakdown of glycogen and hexose phosphates and the aerobic oxidation of pyruvate by the epiphyseal cartilage were studied by the Warburg manometric technique with the use of standard Warburg apparatus using flasks of 15 ml. capacity with centre well and one side chamber. The anaerobic respiration of cartilage was studied in the atmosphere of nitrogen and for aerobic oxidation of pyruvate, O_2 was used as the gas phase. All observations were made at 37°.

Preliminary trials with slices and homogenates of the epiphyseal cartilage of normal young rats showed that homogenates gave results which were not reproducible probably owing to inadequate homogenization of the cartilage. Hence all experiments described in this paper have been carried out with cartilage slices. Utter, Wood & Reiner (1945) had observed that the addition of ATP, diphosphopyridine nucleotide (DPN), Mg^{2+} and nicotinamide to buffered substrate was necessary to evoke maximum glycolytic activity of cell-free extracts and homogenates prepared from the central nervous system. Preliminary experiments showed that the same cofactors favourably influenced the glycolytic activity of cartilage and hence, they were incorporated in the reaction medium in concentrations given below. In experiments with pyruvate, cytochrome *c*, with ATP and DPN, was found to improve the rate of oxidation. The reaction mixtures for glycolysis and aerobic oxidation experiments were made up as follows:

(*a*) *Anaerobic experiment: gas phase, nitrogen*

		ml.
(i)	Na-K phosphate buffer, pH 7·3 (0·03 M)	1·0
(ii)	$NaHCO_3$ (0·36 M)	0·2
(iii)	$MgSO_4$ (0·24 M)	0·1
(iv)	ATP (0·0021 M)	0·1
(v)	DPN (0·005 M)	0·1
(vi)	Nicotinamide (0·6 M)	0·1
(vii)	Fructose 1:6-diphosphate or Glycogen or G 1-P or G 6-P (0·0075 M)	0·2

The total volume was made up to 3·2 ml. with distilled water. Temperature equilibration was obtained by shaking the reaction mixtures for 10 min. before the cartilage slices were tipped into the chamber from the side arm. Readings were taken every 5 min. for half an hour. Enzyme activity was expressed as $Q_{CO_2} = \mu$l. CO_2 liberated/mg. dry wt./hr.

(*b*) *Aerobic experiment: gas phase, oxygen*

		ml.
(i)	Krebs–Ringer bicarbonate buffer without $CaCl_2$ (pH 7·4)	1·0
(ii)	ATP (0·0021 M)	0·1
(iii)	DPN (0·005 M)	0·1
(iv)	Cytochrome *c* (3×10^{-5} M)	0·1
(v)	Sodium pyruvate (0·05 M)	0·3

The total volume was made up to 3·0 ml. with distilled water; 0·2 ml. of 10 % (w/v) aqueous KOH was placed in the centre well. The cartilage slices were added to the main chamber from the beginning. Equilibration time was 10 min. Readings were taken every 20 min. for 1 hr.

The enzyme activity is expressed as $q_{O_2} = \mu$l. of O_2 consumed/100 mg. dry tissue/hr. The results of pyruvate oxidation with the epiphyseal cartilage of normal 4- to 5-week-old rats are given in the last column of Table 2.

Experimental animals. 180 young and healthy albino rats weighing about 45 g. and between 24 and 28 days of age were caged individually and kept in a room where no direct or reflected sunlight could penetrate. All the animals were fed rachitogenic diet (Dikshit & Patwardhan, 1947) with Ca:P as 10:1. The control rats received 25 i.u. of vitamin D/rat/day. At intervals of 5, 10, 15 and 20 days a fixed number of rats in each group was killed, tibial cartilages were dissected out, cut longitudinally in thin slices and immediately used for manometric observations. In rachitic animals the glycolytic activity was estimated on cartilage slices of individual rats since sufficient amount of cartilage could be obtained from each animal, but in case of vitamin D treated controls the cartilage from two animals had to be pooled for each trial. On the other hand, owing to very low oxidative capacity of the cartilage, slices from two animals in the rachitic group had to be pooled for experiments with pyruvate.

RESULTS AND DISCUSSION

Bywaters (1936*a*, *b*), Dickens & Weil-Malherbe (1936), Hill (1940) and Laskin, Sarnat & Bain (1952) have studied the respiratory activity in cartilage with the use of manometric technique. Their results pointed to the fact that the glycogenolytic process in the cartilage was predominantly anaerobic. Even so, the level of glycolytic activity of the epiphyseal cartilage of the tibia observed in the present investigation (Table 1) was much higher than that reported by the above mentioned workers. This might be due to the fact that ATP, DPN and Mg^{2+} were added by us to the reaction medium in suitable concentrations and that glycogen and phosphorylated hexoses were used as substrates. Hill (1940) and Laskin *et al.* (1952) observed a fourfold increase in the oxygen uptake of rabbit and horse articular cartilage when methylene blue was added to the reaction mixture. They also produced evidence to

show that the formation of pyruvate takes place in the metabolic process.

The results in Table 1 indicate that in the initial stages of rachitogenesis, the glycolytic activity of rachitic cartilage with all the substrates tried was considerably higher than that exhibited by the cartilage from vitamin D treated rats. However, as rickets became well established, a fall in glycolytic activity to the level of vitamin D treated controls was witnessed on the 15th day. There were fairly large variations in the values of Q_{CO_2} obtained at different stages of rachitogenesis in comparable animals. On the whole, however, the observed differences between the anaerobic glycolytic activity of cartilage in the rachitic and vitamin D treated animals were not found to be statistically significant. If anything, the values of Q_{CO_2} with G 1-P and G 6-P as substrates were slightly higher after 15 days in rachitic cartilage than in that of vitamin D treated animals.

One can tentatively conclude from these experiments that the mechanism of anaerobic breakdown of glycogen in cartilage is not disturbed in vitamin D deficiency.

The results of experiments dealing with pyruvate oxidation are summarized in Table 2.

As mentioned earlier, q_{O_2} (pyruvate) was very much less than the glycolytic activity displayed by rachitic cartilage. There was a difference in the early stages of rachitogenesis between the endogenous respiration of the cartilage and the rate at which the added pyruvic acid was oxidized. Statistical analysis of the results shows that this difference was more marked in vitamin D treated rats than in deficient animals ($P < 0.001$), as observed on the fifth day after placing the rats on rachitogenic diets. As the rats continued on the diet and with the establishment of rickets the capacity to oxidize pyruvic acid suffered a decrease in rachitic rat cartilage and after 20 days on the rachitogenic diet, the rachitic cartilage seemed to have lost altogether the capacity to oxidize pyruvic acid. On the other hand, in rats treated with vitamin D, pyruvate oxidation remained mostly unimpaired, as would be evident from a comparison of these results with those on cartilage of normal young rats given in Table 2. These results, therefore, clearly indicate that in vitamin D deficiency the rachitic epiphyseal cartilage of rats is unable to oxidize pyruvic acid, thus confirming the suspicions expressed in the earlier paper by Ramalingaswami *et al.* (1954).

Pyruvate oxidation trials were carried out with liver and kidney homogenates prepared according to the method of Green, Loomis & Auerbach (1948) from rachitic and vitamin D protected rats. The results showed that the capacity of these organs to oxidize pyruvate was not impaired in vitamin D deficiency. It appears possible, therefore, that the effect of vitamin D deficiency described in this paper is restricted to bone.

The antirachitic action of citrate demonstrated by Shohl (1937) and the essential similarity of histochemical changes in rachitic cartilage induced under the influence of vitamin D on the one hand and citrate on the other as observed by Ramalingaswami *et al.* (1954) had indicated that in vitamin D deficiency one was more likely to find an arrest or disturbance in the oxidative phase of glycogen metabolism than in the earlier and anaerobic stages of glycogen breakdown. The experiments described here have fulfilled these expectations. It is not yet clear, however, how the orderly progress of glycogen metabolism would be essential to calcification. Two tentative suggestions can be made at this stage. It is possible that the supply of citrate itself is inadequate in rickets owing to a progressive inability of the cartilage to deal with pyruvate. That citrate is of importance to bone can be concluded from the observation of Dickens (1941) that 90 % of citrate in the animal body is to be found in the skeleton. The same author has shown that in rickets citrate in bone is reduced. Further, the relationship between the state of vitamin D nutrition and citrate metabolism has been well brought out by the work of Harrison & Harrison (1952), who found that in

Table 1. *Glycolytic activity of rat epiphyseal cartilage*

		μl. CO_2/mg. dry wt./hr. with substrate (averages only are given)			
		Glycogen	Glucose 1-phosphate	Glucose 6-phosphate	Fructose 1:6-diphosphate
Five days on rachitogenic diet	Without vitamin D (16 rats)	15·1	16·6	13·8	24·1
	With vitamin D in diet (10 rats)	6·3	7·8	7·2	7·1
Fifteen days on rachitogenic diet	Without vitamin D (10 rats)	5·8	6·8	7·6	7·0
	With vitamin D in diet (12 rats)	4·6	3·9	3·5	6·5

Table 2. *Oxidation of pyruvate by epiphyseal cartilage of rat tibiae in rachitic and vitamin D protected animals*

	Rats on rachitogenic diets			Rats on rachitogenic diets and vitamin D			Normal rats on colony diets (8)*
	Five days (32)*	Ten days (12)*	Twenty days (24)*	Five days (32)*	Ten days (12)*	Twenty days (20)*	
	S.E.		S.E.	S.E.		S.E.	S.E.
(*a*) Q_{O_2} with substrate in μl./100 mg. dry tissue/hr.	191·2±16·57	187·4	132·1±15·53	252·5±13·85	288·4	257·5±15·72	284·2±14·41
(*b*) Q_{O_2} without substrate in μl./100 mg. dry tissue/hr.	137·3±10·68	161·3	138·1±10·41	129·2± 8·30	154·7	150·7± 9·72	118·5± 5·86
Q_{O_2} (pyruvate) ($a-b$)	53·9	26·1	−6·0	123·3	133·7	106·8	165·7
Student's '*t*'	2·7358	—	0·3187	7·6409	—	5·8880	10·6479
P	<0·02	—	0·8	<0·001	—	<0·001	<0·001

The significance of differences in Q_{O_2} pyruvate within and between groups on 5th and 20th days is tabulated below:

(1) Rachitic 5th and 20th days	$t=4·6244$ (26)†	*P*, <0·001
(2) Vitamin D 5th and 20th days	$t=0·9296$ (24)	Not significant *P* between 0·3 and 0·4
(3) Rachitic and vitamin D on 5th day	$t=4·4318$ (30)	*P*, <0·001

* Figures in parenthesis indicate the number of animals.

† Degrees of freedom.

rachitic infants the concentration of citrate in serum was significantly lower than in normal infants, and that it was raised on administration of vitamin D. Dixon & Perkins (1952) have shown the existence of enzyme systems, viz. citrogenase, aconitase and isocitric dehydrogenase in bone. It may not be unreasonable to conclude that the formation of citrate is inhibited in rickets, a conclusion of which experimental confirmation is now being sought.

On the other hand, other possible roles which pyruvate oxidation may play in calcification will have to be examined. Much of the energy needed for calcification must be derived from the progressive oxidation of pyruvic acid. Then again, our earlier observations (Ramalingaswami *et al.* 1954) suggested a possible defect in the formation of cartilage matrix in rickets which seems to undergo a qualitative change after treatment with vitamin D. It is possible that the formation of the appropriate matrix may be closely related to pyruvate metabolism in cartilage.

SUMMARY

1. Anaerobic breakdown of glycogen and phosphorylated hexoses and the oxidation of pyruvate by epiphyseal cartilage of the tibiae of rats kept on rachitogenic diets with and without vitamin D has been studied by the Warburg manometric technique.

2. In rachitic rats the glycolytic activity of the epiphyseal cartilage was almost the same as that observed in vitamin D protected rats in comparable experiments.

3. In well established rickets the epiphyseal cartilage of the rat loses the capacity to oxidize pyruvate.

4. The significance of the above findings in the mechanism of calcification has been discussed.

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The Carbohydrate Metabolism of the Isolated Diaphragm of the Rat. Glucose Uptake and Body Size

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Since Gemmill (1940, 1941) and Gemmill & Hamman (1941) have shown that the isolated diaphragm of the rat is a suitable tool for the experimental study of the glucose uptake and glycogen synthesis by muscle tissue, and demonstrated that these processes are stimulated by *in vitro* addition of insulin many investigators have used the method to study the hormonal control of carbohydrate metabolism. More recently, attempts have been made to estimate small amounts of insulin by that method (Groen, Kamminga, Willebrands & Blickman, 1951–2). The experimental conditions under which reproducible results may be expected have been the subject of several studies (Stadie & Zapp, 1947; Krahl & Cori, 1947; Krahl & Park, 1948), but for some time the stimulation of the glucose uptake by insulin could not be repeated in this laboratory. Similar difficulties were encountered by Perlmutter, Weisenfeld & Mufson (1952). Therefore it was felt necessary to re-examine the effect of some factors on the carbohydrate metabolism of the isolated diaphragm of the rat and on the stimulating effect of insulin on its glucose consumption.

The present paper deals with the relation existing between the size of the rats and the uptake of glucose measured. It was found that the uptake of glucose per g. of tissue was high if small rats were used, and low if large rats were used. Possible explanations are presented. Differences between insulin effects obtained with animals of different sizes could not be demonstrated. A preliminary note has appeared elsewhere (Liébecq, 1953). Later work will show the effect of other factors on the extent of the insulin effect.

METHODS

All rats were obtained from Brofo, Zevenaar, Holland, and were of the Epymus Albino Wistar strain. They were fed a mixture of oat flakes (50%), skimmed dried milk (35%), wheat germ (6%), dried yeast (6%), $CaCO_3$ (1·5%), NaCl (1%) and cod-liver oil (0·5%) *ad lib.*, supplemented with fresh vegetables. This diet contains 25–27% protein, 5–6% lipid, 60–65% carbohydrate and 5–6% ash. Rats were fasted 20–24 hr. before use.

The animals were killed by decapitation and the two hemidiaphragms were carefully excised and immediately transferred into two Warburg flasks kept in ice both containing 1 ml. of glucose (1·4 mg./ml.) in Krebs & Henseleit's (1932) bicarbonate medium (but with 6 mg. calcium/100 ml. instead of 10). Excess of Novo insulin (5 μg., i.e. approx. 0·1 unit/ml.) free of hyperglycaemic factor was present in one flask only. The flasks were filled with 95% O_2+5% CO_2 and incubated for 30 min. at 37° while shaken at 140–145 strokes of approx. 5 cm./min., then cooled. For glucose determination, 0·3 ml. samples (in duplicate) were diluted with 5 ml. water and deproteinized by addition of 0·45 ml. 0·3 N-$Ba(OH)_2$ and 0·40 ml. 0·15 M-$ZnSO_4$ (9 ml. of the $Ba(OH)_2$ solution added dropwise should neutralize 10 ml. of the $ZnSO_4$ solution in the presence of 100 ml. water and 10 drops of 0·1% (w/v) phenolphthalein in ethanol). Glucose was determined on 1 ml. of the filtrate, in duplicate, by the method of Nelson (1944) as modified by Somogyi (1952). The hemidiaphragms were blotted on filter paper at the end of the experiment and weighed. The uptake of glucose was calculated in mg./g. of moist tissue/hr.

All solutions were prepared with reagents of analytical grade purity and with glass-distilled water. To remove residual insulin, stainless steel instruments were soaked in a concentrated solution of a commercial preparation of alkaline detergent, pipettes in 2 N-NaOH, then washed carefully and dried in an oven at 120–130°. Warburg flasks were boiled in the solution of alkaline detergent for at least 10 min. before washing.

Table 1. *Uptake of glucose by two hemidiaphragms of rats weighing 50–160 g.*

Figures represent the mean ± S.E.M., the number of experiments being given in parentheses.

Weights of rats (g.)	Without insulin		With insulin	
	Weight of tissue (mg./flask)	Glucose uptake (mg./g./hr.)	Weight of tissue (mg./flask)	Glucose uptake (mg./g./hr.)
50–60	67·4 ± 1·3 (12)	7·42 ± 0·64 (12)	68·3 ± 1·2 (12)	10·37 ± 0·57 (12)
75–85	91·1 ± 2·7 (12)	5·55 ± 0·35 (12)	96·2 ± 2·8 (12)	8·08 ± 0·30 (12)
100–110	123·5 ± 3·4 (12)	4·01 ± 0·20 (12)	127·0 ± 3·7 (11)	6·12 ± 0·18 (11)
125–135	157·9 ± 4·8 (6)	3·58 ± 0·18 (6)	159·0 ± 5·2 (6)	5·08 ± 0·27 (6)
150–160	189·3 ± 5·1 (6)	3·33 ± 0·11 (6)	189·8 ± 4·9 (6)	4·87 ± 0·17 (6)

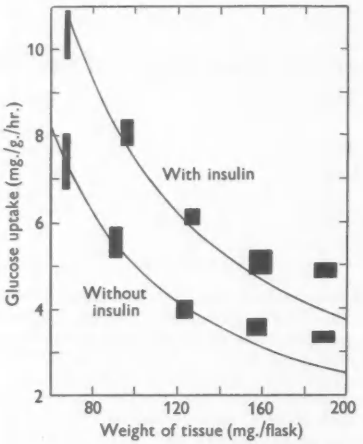

Fig. 1. Uptake of glucose by two hemidiaphragms of five groups of rats weighing 50–60, 75–85, 100–110, 125–135 and 150–160 g., respectively. The black quadrangles represent the mean weights of tissue and glucose uptakes ± S.E.M. The upper and lower curves are rectangular hyperbolas calculated for a 'weight × glucose uptake' product of 750 and 500, respectively.

When applied, the test for significance of differences between means was Student's t test (see Snedecor, 1946).

RESULTS

In the first set of experiments, five groups of rats were used, weighing 50–60, 75–85, 100–110, 125–135 and 150–160 g., respectively. The hemidiaphragms of two rats were used in each experiment. One Warburg flask containing one hemidiaphragm from each rat was incubated without insulin, the other flask containing the remaining hemidiaphragms was incubated with insulin: in this way, left and right hemidiaphragms were equally distributed between the control and insulin-containing flasks. The thickness of the pieces of tissue was not determined in all cases but the thinnest (among rats weighing 50–60 g.) were approx. 0·25 mm., the thickest (among heavy rats) approx. 0·55 mm. It is clear from the results given in Table 1 that thin hemidiaphragms of small rats take up much more glucose than the thick hemidiaphragms of larger rats. For the first three groups of rats, the actual amount of glucose taken up (per manometer flask, not per g./hr.) was practically independent of the amount of tissue per flask: 65–125 mg. of tissue took up 0·24–0·26 mg. of glucose in 30 min. without insulin, and 0·35–0·39 in the presence of insulin. Fig. 1 shows that, with amounts of tissue up to 120–130 mg. per flask, the experimental values approximately follow the course of rectangular hyperbolas: the product 'glucose uptake (mg./g./hr.) × weight of tissue (mg./flask)' is essentially constant. Indeed, this is not too surprising, since the diffusion of glucose, oxygen and possibly other metabolites depends on the thickness of the preparation. However, the effect of tissue thickness on the course of the reaction is so important that two groups of results are not comparable unless the mean weights of tissue of the two groups are really identical. Thus, it is not permissible to assess the effect of insulin by comparing directly, for instance, the two values of line 2, Table 1 (5·55 and 8·08 mg./g./hr.), since the mean weights of tissues are in these cases 91·1 and 96·2 mg./flask, respectively. The relation existing between weight of tissue and glucose uptake makes it possible to predict what the mean uptake would be if the mean weight of tissue were 93·7 mg./flask in both cases:

$$91{\cdot}1 \times 5{\cdot}55 = 93{\cdot}7 \times x; \quad x = 5{\cdot}40.$$
$$96{\cdot}2 \times 8{\cdot}08 = 93{\cdot}7 \times y; \quad y = 8{\cdot}30.$$

The stimulating effect of insulin on the uptake of glucose by hemidiaphragms of rats weighing 75–85 g. is thus: 8·30 − 5·40 = 2·90 mg. extra glucose taken up/g. of tissue/hr., i.e. a 54% increase. Statistical analysis however requires that all corrections be applied to individual values before calculation of the means. If this is done (see Table 2), all mean values are slightly modified and the standard errors are generally diminished. From such comparable figures, the effect of insulin on the uptake of glucose may be calculated. If expressed as extra glucose uptake, the maximum effect of insulin was obtained with the thinnest hemi-

Table 2. *Effect of insulin on the uptake of glucose by hemidiaphragms of rats weighing* 50–160 *g.*

Weights of rats (g.)	Weight of tissue* (mg./flask)	Glucose uptake†		Effect of insulin‡	
		Without insulin (mg./g./hr.)	With insulin (mg./g./hr.)	Extra uptake of glucose (mg./g./hr.)	Stimulation (%)
50–60	67·9	7·23±0·61 (11)	10·49±0·56 (11)	3·26±0·39 (11)	50·6± 8·4 (11)
75–85	93·7	5·36±0·30 (11)	8·32±0·28 (11)	2·96±0·16 (11)	57·2± 4·2 (11)
100–110	125·2	3·96±0·22 (11)	6·22±0·11 (11)	2·26±0·20 (11)	61·1± 7·6 (11)
125–135	158·5	3·56±0·16 (6)	5·08±0·23 (6)	1·52±0·33 (6)	44·9±11·0 (6)
150–160	189·5	3·32±0·13 (6)	4·89±0·26 (6)	1·57±0·29 (6)	48·3± 9·4 (6)

* Mean of the weights given in Table 1 for the experiments with and without insulin.

† Compare with the data of Table 1.

‡ In all cases, the effect of insulin is statistically significant ($P < 0·01$); differences between insulin effects expressed as extra uptake of glucose are also significant but differences between insulin effects expressed in percentage stimulation are not.

Table 3. *Uptake of glucose by* 1–4 *hemidiaphragms of rats weighing* 75–85 *g.*

All individual values were recalculated as if the weights of tissue per flask were really multiples of 46·9 mg., mean weight of a single hemidiaphragm in this set of experiments. In all cases, the effect of insulin is significant ($P < 0·01$). Glucose uptake in the presence of insulin and insulin effects (expressed as extra uptake of glucose or in percentage stimulation) are significantly lower ($P < 0·01$) if four hemidiaphragms are incubated together instead of two.

No. of hemidiaphragms per flask	Glucose uptake		Effect of insulin	
	Without insulin (mg./g./hr.)	With insulin (mg./g./hr.)	Extra uptake of glucose (mg./g./hr.)	Stimulation (%)
1	5·71±0·57 (11)	7·84±0·34 (12)	1·98±0·51 (11)	47·1±16·0 (11)
2	5·35±0·28 (12)	8·26±0·26 (12)	2·96±0·16 (11)	57·1± 4·2 (11)
3	4·88±0·13 (8)	7·75±0·34 (8)	2·48±0·35 (6)	51·9± 8·3 (6)
4	4·94±0·07 (8)	6·83±0·19 (8)	1·90±0·16 (6)	38·4± 3·1 (6)

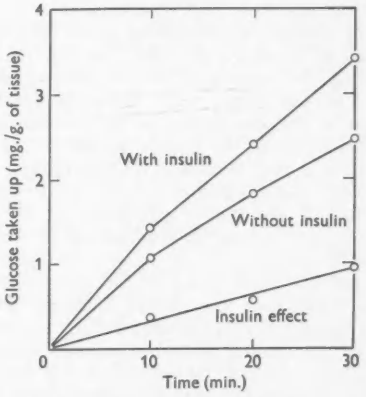

Fig. 2. Uptake of glucose by four hemidiaphragms of rats weighing 75–85 g.

diaphragms; but if considered as the stimulation of a basic glucose uptake, and therefore expressed in percentage stimulation, the maximum effect of insulin was obtained with rats weighing 100–110 g.: differences between insulin effects expressed in this way were however not statistically significant. Reasons for preferring the second mode of expression will be given in the Discussion. The results of Table 2 also show that the individual values of glucose uptake are very scattered when hemidiaphragms of small rats are used.

In a second set of experiments, material from rats all weighing 75–85 g. was used; one, two, three or four hemidiaphragms, respectively, were placed in a flask and incubated, care being taken to ensure equal distribution of left and right hemidiaphragms between control and insulin-containing flasks. In this set of experiments, the mean weight of a single hemidiaphragm was 46·9 mg. Using the correction outlined above, all individual values were recalculated as if the weights of tissue per flask were multiples of 46·9 mg. The results show (Table 3) that the uptake of glucose is practically the same in each case except when four hemidiaphragms are incubated together in the presence of insulin. This lower figure, together with the corresponding lower effect of the added insulin, might have been due to a limiting effect of the glucose concentration which is known to affect both the uptake of glucose and the synthesis of glycogen (Gemmill & Hamman, 1941; Hechter, Levine & Soskin, 1941; Stadie & Zapp, 1947; Riesser, 1947; Mentha & Voegtli, 1947) since the final glucose concentration had fallen, in this case, to a mean value of 0·76 mg./ml. against the initial value of 1·4 mg./ml. An experiment was carried out to follow the course of the reaction during shorter periods of incubation (10, 20 and

30 min.) and four hemidiaphragms were incubated to produce a sufficient uptake of glucose; it was found however, that (see Fig. 2) the extra uptake of glucose produced by addition of insulin was not diminished in rate after 20 min. as it would have been if low glucose concentration had limited the glucose consumption. It is possible that shaking is not efficient enough when four pieces of diaphragm are incubated together in the same flask; Brown, Park, Daughaday & Cornblath (1952) have shown that the rate of shaking is critical.

DISCUSSION

It is well known that the relative metabolic rate of large animals is lower than that of smaller animals and that the energy exchange is more or less proportional to the surface area rather than to the weight of the animal. From a comprehensive study of the respiration of isolated tissue preparations, Krebs (1950) concluded that for most tissues, the Q_{O_2} changed much less with the body weight than the rate of basal heat production of the intact animal; Krebs believes that the differences in the total rate of heat production in animals of different size are mainly due to variations in the Q_{O_2} of the musculature: this conclusion was reached by exclusion and not by direct evidence. Von Bertalanffy & Pirozynski (1951) and von Bertalanffy & Estwick (1953), who used slices of tissues or isolated diaphragms of the rat, recently showed that the Q_{O_2} of isolated tissues is higher when young animals are used. Statistical evaluation of their data for diaphragm demonstrates a negative correlation between the rate of respiration and the body size: although these results, as well as those of Kayser, Le Breton & Schaeffer (1925), seem to corroborate Krebs's conclusion, it is felt that the evidence so far presented is not satisfactory since the variable thickness of the preparations was not considered: diaphragms of rats weighing more than 100 g. are definitely too thick for reliable manometric measurements. It is interesting to note in this respect that the slope of the regression line is negligible ($-0{\cdot}065$, with a correlation coefficient of $-0{\cdot}66$ only) when fibres of skeletal muscle are used instead of diaphragms where the slope of the regression lines ranged from $-0{\cdot}15$ to $-0{\cdot}26$ according to the medium used. Therefore, it is felt reasonable to assume that the metabolism of the isolated diaphragm is practically independent of the size of the rats used. In contrast with this conclusion, the uptake of glucose is highly dependent upon the size of the rats used: calculated in the same way but using glucose uptakes instead of oxygen uptake, the slope of the regression line shows a very marked negative correlation between glucose uptake and body size, whether insulin be present or not ($-0{\cdot}72$ and $-0{\cdot}77$, respectively, with correlation coefficients of $-0{\cdot}98$ and $-0{\cdot}99$), and it is extremely unlikely that this reflects a reduction of the carbohydrate metabolism in the isolated diaphragm of large animals.

The hemidiaphragms of large rats could differ from those of small rats in three ways: (1) their true activity may be different, (2) their surface is larger, but not very much so apparently, and (3) they are thicker. The first possibility is not supported by the arguments developed in the first part of this discussion. It is clear from the experimental results reported in Table 3 that the relative uptake of glucose (glucose taken up/g. of tissue) is essentially unaffected by the number of hemidiaphragms used when they are of about the same size, i.e. the absolute uptake of glucose is proportional to the number—or to the surface—of the hemidiaphragms. The low glucose uptakes by hemidiaphragms of larger rats may probably be best explained by differences in rates of diffusion in preparations of varying thickness; the possibility that an inhibitor present in the hemidiaphragms diffuses out more easily from thin preparations may also have to be considered. The results presented support the view that the absolute uptake of glucose is apparently proportional to the surface of the diaphragms—independent of the thickness of the preparation—rather than to the weight of tissue. This would mean that only the superficial layers consume glucose, possibly because the superficial layers only are in contact with enough glucose. The fact that the centre of the preparation may be lacking oxygen cannot explain these results since the uptake of glucose is not diminished in an atmosphere of nitrogen, although the stimulating effect of insulin nearly disappears (Walaas & Walaas, 1952).

The stimulating effect of insulin could be demonstrated and was highly significant in all cases ($P < 0{\cdot}01$) but its magnitude varied slightly with the type of preparation used (see Table 2); the differences observed were however not statistically significant. When considering the results obtained without added insulin, there is probably no need to take into account the amount of insulin possibly present in the hemidiaphragms. If we assume that insulin is evenly distributed between plasma and extracellular fluid, and, since the concentration of insulin in normal (human) plasma was found to be of the order of 1×10^{-4} units/ml. (i.e. approx. 5×10^{-3} μg./ml.) by Groen *et al.* (1951–2) using the diaphragm technique and by Bornstein (1950*b*) who measured the hypoglycemia produced by injection of insulin to alloxan-diabetic, hypophysectomized, adrenalectomized rats (Bornstein, 1950*a*), the amount of insulin present in 50–200 mg. of diaphragm would be of the order of $1\text{–}5 \times 10^{-6}$ units: this amount, diluted by the addition of 1 ml. of glucose-contain-

ing medium would be unable to produce any measurable stimulating effect (Krahl & Cori, 1947; Groen *et al.* 1951–2).

There is no possible comparison between the extra oxygen uptake produced by the addition of a substrate to a slice of surviving tissue and the extra glucose uptake produced by the addition of insulin to an isolated tissue incubated in a glucose-containing medium. In the first case, the control without substrate is due to the oxidation of various endogenous metabolites and the stimulating effect of the added substrate on the respiration rate is generally the result of a different and additional reaction. In the case of insulin, there are no reasons to believe that the extra glucose uptake is produced by a different enzymic system. In fact there are good reasons for believing that the glucose disappears as the result of a chain of reactions of which the first is catalysed by hexokinase. Unpublished measurements from this laboratory show that the hexokinase activity of water extracts of rat skeletal muscle reinforced with Mg^{2+} and adenosine triphosphate corresponds to a glucose uptake of approx. 15 mg./g./hr., i.e. 2–3 times as much as the values found for the isolated diaphragm. From the figures of Long (1952) and the calculations he made of the results of Stadie & Haugaard (1949), it may be inferred that the activity of whole homogenates of rat skeletal muscle would be of the order of 30 mg./g./hr. Although the activity of water extracts of muscle measured by other authors are not as high as ours (Stadie & Haugaard (1949) found approx. 11 mg./g./hr. and Gerritsen (1951) only 8–9 mg./g./hr.) there is no doubt that the uptake of glucose by the isolated diaphragm in the absence of added insulin is not limited by the rate of the hexokinase activity; the highest values found in the presence of insulin are still below the level of hexokinase activity found by us in muscle extracts and by Long (1952) in muscle homogenates. Therefore it is felt preferable to express the effect of insulin as the stimulation of the control glucose uptake and not as an extra glucose uptake. Rats weighing about 100 g. will provide hemidiaphragm preparations most sensitive to insulin and more accurate determinations will be made with such rats. Data on the uptake of glucose by homogenates of rat diaphragms have not been published; an approximate value of 10–12 mg./g./hr. is given by Krahl (personal communication).

SUMMARY

1. Diaphragms from rats of varying weights were used for measurements of the rates of glucose uptake and their stimulation by insulin.

2. The reaction has been studied as a function of the amount of tissue used per manometer flask. The uptake of glucose was found to be proportional to the number of hemidiaphragms used when they were of about the same size, but thin hemidiaphragms of small rats took up as much glucose as thick hemidiaphragms of heavy rats; the product 'glucose uptake/g. of tissue × weight of tissue/flask' was found to be essentially constant. This is taken as evidence that the superficial layers of the preparations take up much more glucose than the centre of the preparations, although this cannot be the only explanation.

3. The simple relation between the uptake of glucose/g. of tissue and the weight of tissue/flask makes it possible to predict by calculation the uptake of glucose by different amounts of tissue; the stimulating effect of insulin may only be assayed from a comparison of the glucose uptake in the absence and in the presence of added insulin, if the mean weight of tissue/flask is the same in the two sets of experiments. Differences between the size of the insulin effect measured with thin or thick preparations were not statistically significant.

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The *N*-Terminal Amino Acid Residues of Gelatin

1. INTACT GELATINS

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During the past five or six years there has been a considerable revival of interest in the biochemistry and physical chemistry of collagen and gelatin (Randall, 1953; Pouradier & Venet, 1950, 1952; also others). Gelatin may be defined as the protein derived from collagen by irreversible procedures which render the collagen water-soluble. The irreversibility differentiates gelatin from 'soluble collagen'. Gelatin is not homogeneous within any one sample, and different samples differ in important properties and structural characteristics (e.g. molecular weights, molecular-weight distribution, isoelectric point, viscosity of solutions, rigidity of gels). The possibility arises that these differences may be due in part at least to the method of manufacture, of which there are two main procedures, the acid process and the alkali process (Ward, 1951; Ames, 1952) or to the type of collagenous tissue used. There are three principal sources of collagen used commercially: skin, bone and sinew. This fact was not always appreciated in the past by those unfamiliar with the industrial process and has often led to difficulties in correlating results of different workers engaged on similar research.

The nature of the bonds broken when collagen is converted into gelatin is still a matter of speculation, and the problem is evidently connected with theories of the forces which bind individual protein chains into collagen fibrils. Recent views put forward include hydrogen bonding (Gustavson, 1950), the presence of chondroitin sulphuric acid (Jackson, 1953) and a —CO—NH—CO— linkage (Ames, 1952). Bowes & Moss (1951), using Sanger's 1-fluoro-2:4-dinitrobenzene (FDNB) technique, have shown that, while native collagen appears to have no *N*-terminal end groups, gelatin gives small amounts of *N*-terminal aspartic acid, glutamic acid, glycine and threonine. It appears, therefore, that the rupture of peptide bonds may be involved in the conversion of collagen into gelatin.

The purpose of the present investigation was to ascertain any gross differences in *N*-terminal residues between gelatins with different collagenous precursors or with the process of manufacture used.

The *N*-terminal-residue method gives useful approximations for the number-average molecular weights of gelatin samples, based on the assumption that the protein comprises single polypeptide chains each with one *N*-terminal residue.

As Sanger's end-group technique has been applied to an increasing number of proteins, certain workers have reported that not all of the ϵ-amino groups of lysine and hydroxylysine residues are available to react with FDNB, although in some instances these groups are available to nitrous acid (Salo, 1950) or keten (Porter, 1948). Porter found that more than one-third of ϵ-amino groups of β-lactoglobulin and of several serum globulins failed to react with FDNB when these proteins were in the native state. On denaturation all the groups became available to the reagent. It was suggested, therefore, that the configuration of the polypeptide chain in the native protein could not accommodate the bulky 2:4-dinitrophenyl (DNP) group. A similar phenomenon has been reported with collagen and gelatin, although there is disagreement over the extent of availability of ϵ-amino groups; Bowes & Moss (1951) found about 50 % availability with native collagen and gelatin, while Sykes (1952) reported about 75–80 % availability with hide powder. It was therefore of interest to examine DNP-gelatins for their content of N^{ϵ}-DNP-lysine.

MATERIALS AND METHODS

Gelatins. Eleven high-grade gelatins were chosen to give a wide cross-section of types, and these are described in Table 1. Eight of these were commercial samples; *F* and *J* were laboratory preparations kindly given by Dr W. M. Ames; *L* was prepared from isinglass, made from Beluga sturgeon (*Huso huso*) swim bladder which was kindly supplied by James Vickers and Co.

Reaction with FDNB. The method used was essentially that of Sanger (1945). It was found, however, that gelatin was sensitive to the rapid substitution which occurred in the presence of ethanol, yielding a viscous liquid identical in appearance with the DNP-derivative of a highly degraded gelatin. Ethanol was therefore excluded from the reaction mixture. A solution of gelatin (2 g.) in water (100 ml.) and 8% (w/v) $NaHCO_3$ (100 ml.) was shaken with excess FDNB (4 ml., 5·7 g.) for 24 hr. The solution was initially at 35°, decreasing to room temp. after about an hour. Neutralization with dilute hydrochloric acid deposited a yellow gel, which was dried over P_2O_5 and NaOH, crushed in a mechanical disintegrator and washed with acetone until no further yellow colour was removed. The powder was dried at 105° and allowed to equilibrate with the atmosphere. Samples were then taken for moisture, ash and end-group determinations. The mother liquors contained only a negligible quantity of soluble DNP-peptides and so were ignored. DNP-gelatin was similar to the unsubstituted gelatin in having a moisture content which was very sensitive to atmospheric humidity. The ash content was largely NaCl formed during neutralization. It was found that this salt had no deleterious effect on the stability of DNP-amino acids during the hydrolysis of DNP-gelatin and a similar lack of effect was found with NaF and $MgSO_4$. Consequently, it was unnecessary to remove the salts by prolonged washing with water and hence the risk of removing any soluble DNP-peptides was eliminated. This precaution was particularly important when dealing with acid-processed gelatins whose DNP derivatives were markedly water-soluble.

Moisture. About 100 mg. of material was heated at 105° for 16 hr. in a micro moisture dish.

Ash. Samples (about 200 mg.) were ashed in Pt dishes at 550° for 16 hr.

Hydrolysis. DNP-gelatin (about 500 mg.) was heated with 5·5 N-HCl for 16 hr. in sealed tubes at 100°. These conditions lead to destruction of free DNP-proline and DNP-hydroxyproline (Porter & Sanger, 1948). It was therefore necessary to choose alternative hydrolysis conditions when attempting to detect these amino acids as *N*-terminal residues. Porter & Sanger showed that the complete destruction of the DNP-derivatives may be avoided by employing 12 N-HCl at 105° for 16 hr., giving a recovery of 50%. Bowes & Moss (1953) recovered 20% of DNP-proline using similar conditions in the presence of collagen. The present author could only recover 15% of DNP-proline using these hydrolysis conditions in the presence of gelatin. Consequently, in attempts to detect DNP-proline and DNP-hydroxyproline in DNP-gelatin hydrolysates, about 4 g. of DNP-gelatin were subjected to the conditions of Porter & Sanger, to make it possible to detect them with certainty. The DNP derivatives of acidic and neutral amino acids were removed from the hydrolysate by extraction with ether.

Identification. The DNP-amino acids were separated chromatographically on buffered Celite columns (Celite 545, Johns-Manville Co., London) using essentially the method described by Perrone (1951).

Two columns were required. The DNP-amino acid mixture was introduced firstly to the top of a column of 4·5 g. Celite (0·5 ml. stationary phase/g. Celite) buffered at pH 4 with phosphate–citric buffer (54 ml. 0·2 M-Na_2HPO_4 + 46 ml. 0·2 M citric acid), the solvent being $CHCl_3$–ether (90:10 by vol.). All artifacts and DNP-amino acids, with the exception of DNP-glutamic acid (*R*, 0·4), DNP-threonine (*R*, 0·2), DNP-serine (*R*, 0·1), DNP-aspartic acid (*R*, 0·05) run fast on this column. These four derivatives separate well and their elution may be speeded up by the following procedure. When DNP-glutamic acid has been run off, the solvent is changed to $CHCl_3$–ether (50:50 by vol.); when DNP-threonine has been run off, the solvent is changed to $CHCl_3$–ether (20:80 by vol.); when DNP-serine has been run off the solvent is changed to $CHCl_3$–ether (5:95 by vol.). When DNP-aspartic acid was removed, the column was washed through with about 10 ml. of $CHCl_3$ in preparation for the

Table 1. *Origin and properties of gelatin specimens*

Key	Process	Origin	Jelly strength* g.	Viscosity† cp.	Reduced viscosity‡
A	Alkali	Ox bone	217	7·05	0·59
B	Alkali	Ox bone	186	5·33	0·45
C	Alkali	Calfskin	208	7·65	0·60
D	Alkali	Calfskin	250	7·40	—
E	Alkali	Oxhide	191	7·79	0·56
F	Alkali	Ox sinew	274	7·75	—
G	Acid	Pigskin	235	7·31	0·43
H	Acid	Pigskin	221	5·62	0·39
J	Acid	Ox sinew	276	4·60	—
K	Acid	Ox bone	242	4·15	0·34
L	Acid	Swim bladder	—	—	—

* Essentially a measure of gel rigidity. Determined according to British Standards Institution (1944) 757 at 6·67% (w/v).

† Determined according to British Standards Institution (1944) 757 at 6·67% (w/v) and 40°.

‡ The reduced viscosity, as defined by Stainsby, Saunders & Ward (1954) and expressed as $1/c \ln \eta_{rel.}$, was determined at pH 7 in M-NaCl at 35°, the gelatin concentration, *c*, being 0·5% (w/v). Under these conditions, small changes in pH or ionic strength produce only very small changes in the value of $1/c \ln \eta_{rel.}$. The author would like to thank Dr G. Stainsby for these determinations.

Table 2. *N-terminal amino acid residues of various gelatins*

	moles/100000 g. gelatin (moisture and ash free)										
	Alkali-processed						Acid-processed				
	A Ossein	*B* Ossein	*C* Calf	*D* Calf	*E* Oxhide	*F* Sinew	*G* Pigskin	*H* Pigskin	*J* Sinew	*K* Ossein	*L* Isinglass
Glycine	0·95	0·98	0·83	0·80	1·27	1·00	0·74	0·59	0·96	0·72	0·25
Serine	0·24	0·23	0·19	0·19	0·24	0·23	0·10	0·05	0·04	0·06	0·05
Threonine	0·11	0·10	0·11	0·11	0·14	0·14	0·08	0·03	0·03	0·05	0·03
Alanine	0·13	0·17	0·11	0·10	0·20	0·17	0·24	0·18	0·23	0·16	Nil
Aspartic acid	0·10	0·12	0·11	0·08	0·14	0·15	0·13	0·06	0·02	0·04	0·03
Glutamic acid	0·14	0·13	0·10	0·10	0·15	0·12	0·07	0·04	0·04	0·03	0·04
Others	0·01	0·14	0·12	0·12	0·20	0·08	0·19	0·19	0·11	0·05	Nil
Total	1·68	1·87	1·57	1·50	2·34	1·89	1·55	1·14	1·43	1·11	0·40
Number-average mol.wt. $\times 10^{-3}$	60	53	64	67	43	53	65	87	70	88	250

Table 3. *Availability of ε-amino groups to FDNB in various gelatins*

Key	ε-Amino group availability (%)
A	100
B	97
C	97
E	96
G	97
H	88
K	90

following procedure. The fast band previously obtained from this column was evaporated to dryness and taken up with 0·5 ml. $CHCl_3$. When reintroduced to the top of the column and eluted with $CHCl_3$, DNP-glycine (*R*, 0·5) separated well from the faster-moving material. This last, after evaporation to dryness, was dissolved in 0·5 ml. $CHCl_3$ and run on a 3 g. Celite column buffered at pH 7·1 (83 ml. 0·2 M-Na_2HPO_4 + 17 ml. 0·2 M citric acid), using $CHCl_3$ initially as the moving phase, until the artifacts (2:4-dinitroaniline and 2:4-dinitrophenol) were removed. The eluent was then changed to ether, enabling the separation of DNP-phenylalanine (*R*, 0·6), DNP-valine (*R*, 0·3) and DNP-alanine (*R*, 0·07). When only DNP-alanine remains on the column, its elution may be speeded up by adding 2 drops of glacial acetic acid to 10 ml. of the solvent. The proportion of aqueous and organic-solvent mixtures used in the preparation of these two-phase liquid mixtures was not found to be critical for the separations of the DNP-amino acids associated with gelatin. The various bands were compared with authentic samples of DNP-amino acids under identical conditions.

Many workers, and Mills (1952) in particular, have noted the adverse effects of daylight on the stability of DNP-amino acids. An important feature of this method of separation is the speed of operation, and the eight *N*-terminal residues associated with gelatin may be isolated within 2 hr. Each DNP-amino acid solution collected was taken to dryness on the rotary evaporator (Partridge, 1951) and estimated colorimetrically in 1% (w/v) $NaHCO_3$ solution with the Spekker absorptiometer (Hilger and Watts Ltd., London) using Ilford 601 filters (Ilford Ltd., London) at 430 mμ. Confirmation of the identity of the DNP-amino acids was effected by de-arylation according to the method of Lowther (1951). The DNP-amino acid was heated in aqueous NH_3 (sp.gr. 0·880) in sealed tubes at 100°. The free amino acid thus obtained was identified on paper chromatograms alongside authentic samples.

ε-Amino groups of lysine. About 10 mg. of DNP-gelatin was hydrolysed with 5·5 N-HCl for 16 hr. in sealed tubes at 100°. The hydrolysate was extracted with ether and the aqueous liquors were evaporated to dryness over P_2O_5 and NaOH. The residue was taken up with methyl ethyl ketone–ether (2:1 by vol.), which had been previously saturated with water, until no further yellow colour was extracted. The extract was introduced to the top of a Celite column buffered at pH 7·1 (83 ml. 0·2 M-Na_2HPO_4 + 17 ml. 0·2 M citric acid) using methyl ethyl ketone–ether (2:1 v/v) as the organic-phase eluent.

The column showed 3 bands: a fast artifact (*R*, 1·2), N^{ϵ}-DNP-lysine (*R*, 0·2) and a slow band (*R*, 0·1), presumed to be N^{ϵ}-DNP-hydroxylysine. This last band has not been confirmed but occurs in such proportions as to give a colour density relative to that of the N^{ϵ}-DNP-lysine as 1:4·5, whereas the proportion of hydroxylysine to lysine in gelatin is reported as 1:4·2 (Chibnall, 1946). Calculations of the recovery of N^{ϵ}-DNP-lysine from DNP-gelatin (after allowing for 30% decomposition during hydrolysis) are based on a lysine content of 4·3 g./100 g. gelatin (Chibnall, 1946). This value is in good agreement with a value of 4·3 g. for gelatin using the technique of Moore & Stein (1951) (Dr Eastoe, private communication) and with a value of 4·18 g. for hide collagen (Salo, 1950).

RESULTS

In all samples of gelatins examined, the principal *N*-terminal residues have been found to be glycine, serine, threonine, aspartic acid, glutamic acid and alanine. Small amounts of valine and phenylalanine and possible traces of others are also found and are listed collectively as 'others' in Table 2.

It was found that, for DNP-amino acids occurring in small amounts, agreement between duplicates was within 5–15%. Agreement for DNP-glycine was generally to within 5%. It is probable, therefore, that the figure for total *N*-terminal residues, and consequently for the number-average molecular

weights calculated from this total, is of similar reproducibility.

ε-Amino-group availability: Values, shown in Table 3 correspond to almost complete reaction between the ε-amino groups situated on lysine residues and FDNB.

DISCUSSION

Glycine is the predominant *N*-terminal residue of gelatin and accounts for at least half of the total residues. No gelatin has been found which is an exception to this rule. Again, all gelatins so far examined, whatever their origin or process of manufacture, clearly show the presence of *N*-terminal serine, threonine, aspartic and glutamic acids and alanine residues as well as traces of others. Differences in the proportions of these subsidiary residues, however, are detected. These differences depend upon whether the gelatin was obtained by acid or alkali pretreatment. The hydroxyamino acids, serine and threonine, and the dicarboxylic amino acids, aspartic acid and glutamic acid, all occur in much larger proportions in alkali-processed materials. On the other hand, the proportion of alanine tends to be larger in acid-processed gelatin. The alkali process hydrolyses a number of side-chain amide groups to free carboxyl groups. The proportion of altered groups will depend upon the intensity of pretreatment. Thus a collagen which has received prolonged alkali treatment will yield a gelatin of isoelectric point about pH 4·8. Less intensive treatment yields gelatins of isoelectric point between this value and about pH 5·5. Gelatins obtained by the acid process generally have the isoelectric point in the region of pH 9. Hence, the two processes yield gelatins of slightly different chemical constitution, differences which could possibly modify the lability of different links of the peptide chain over and above the inherent differences in lability to acid and alkali of the various peptide bonds. No significant differences of *N*-terminal residues have been found which are attributable to the origin of the collagenous precursor. There is evidence from X-ray diffraction studies on collagens of different origins (Astbury, 1940) for a fundamental structural identity. Amino acid analysis (Neuman, 1949; Neuman & Logan, 1950) also shows that gelatins derived from hide, bone and sinew by the same process are of similar chemical composition. Table 2 indicates, however, that in the transformation of collagen to gelatin certain peptide bonds are broken and the nature of some of these bonds will depend upon the process employed. The high-grade alkali-processed gelatins examined showed one mole of *N*-terminal residue in approx. 60000 g. of gelatin the range (excluding sample *E*) being 53000 to 67000. These are number-average values of the molecular weight of a molecularly heterogeneous system and the samples may well contain fractions ranging from one-quarter to 4 times this value (Pouradier & Venet, 1950; Stainsby *et al.* 1954). The mean values are in reasonable agreement with those of Pouradier & Venet (1950), who, using similar types of gelatin, obtained values of 61000 to 67000 by osmotic-pressure measurements. The agreement supports the single-chain structure for gelatin.

The molecular weights calculated for the acid-processed gelatins are somewhat higher than for alkali-processed, corresponding to the detection of fewer *N*-terminal residues. Further considerations, however, suggest a cautious approach to these values (Table 2). A discrepancy with these samples is found in the reduced viscosity values (Table 1), these being substantially lower than might be expected from the values for alkali-processed gelatins. It will, however, be necessary to await physical measurements of the molecular weights, before these results can be explained.

Note added in Proof. The recent paper of Grassmann, W. & Hörmann, H. (1953), *Hoppe-Seyl. Z.* **292**, 24, reports both the *N*-terminal and *C*-terminal groups of gelatin. The *N*-terminal groups correspond closely with those observed in the present work.

SUMMARY

1. The *N*-terminal residues of a number of gelatins have been determined using Sanger's fluorodinitrobenzene technique. Glycine was found to be the predominant end group. Serine, threonine, alanine, aspartic acid and glutamic acid residues also occurred.

2. The number-average molecular weight of these samples was calculated assuming one *N*-terminal residue in each molecule. Average values of 60000 were in agreement with those obtained by osmotic-pressure methods and support the view of a non-branching peptide chain.

3. The method has been used to determine the lysine residues of gelatin. Generally, over 90% of the ε-amino groups of lysine were available to the reagent.

The author is greatly indebted to a number of people for helpful advice and for certain facilities. He would particularly like to acknowledge thanks to Dr S. M. Partridge of the Low Temperature Station for Research in Biochemistry and Biophysics, Cambridge, and Dr W. Middlebrook of Leeds University. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

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The *N*-Terminal Amino Acid Residues of Gelatin

2. THERMAL DEGRADATION

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Gelatin in solution undergoes changes in the presence of proteinases or at elevated temperatures, especially at acid or alkaline pH values, to which the general term 'degradation' is given. The effect of degradation in reducing the solution viscosity, and in impairing the gel-forming power, is so readily demonstrated for gelatin, as compared to other proteins, that it has received considerable study. The change in physical properties has frequently been used as a measure of the extent of the degradation reaction (Sheppard & Houck, 1930, 1932; Pouradier & Venet, 1952 and previous papers; Gerngross & Brecht, 1922; Ames, 1947). In view of the uncertain relationships between such properties and the rate of bond breaking the results obtained must be looked on as only empirical, although of considerable practical value.

The degradation of gelatin gives rise to an increase of α-amino groups and this property has been used for assessing the degree of hydrolysis. Northrop (1921*a*, *b*) has used the formol-titration method. Greenberg & Burk (1927) have employed the Van Slyke nitrous acid method. The predominance in gelatin of ε-amino groups of lysine and hydroxylysine residues would make small changes in α-amino groups difficult to detect by these techniques (the proportion of α- to ε-amino groups in material of molecular weight 50000 is 1:20). In addition, neither method will distinguish between α-amino groups formed by rupture of peptide bonds and any other amino groups such as would be involved in the possible conversion of an arginine residue into ornithine. These methods have been mainly of value in studying extensive degradation where the number of α-amino groups released has become large.

The *N*-terminal residue method as described in the preceding paper (Courts, 1954) is likely to be of value in following the course of protein degradation, especially in the early stages where preferential breaking of labile bonds may occur. Thus the identification of the *N*α-2:4-dinitrophenylamino acids would give some information as to the nature of the weaker peptide bonds and also a measure of the extent of hydrolysis. This paper reports some experiments on the controlled thermal degradation of gelatin using the *N*-terminal residue method. The theoretical considerations arising from the results are discussed and related to the physical properties of the gelatins obtained.

MATERIALS AND METHODS

Gelatin. A de-ionized ossein gelatin of isoelectric point pH 5·1 was used. This was prepared from bone collagen and characterized by its Bloom jelly strength (6·67% concentration), 217 g., and viscosity (40° and 6·67% concentration) 7·05 centipoise.

Bone collagen. This was prepared by the method of Eastoe & Eastoe (1954). I am indebted to Dr J. E. Eastoe for a gift of this material.

Technique of degradation. The extent of protein hydrolysis is determined by time and temperature of heating and by pH of solution. Each of the three was varied in turn while the other two were kept constant. Degradations were carried out in sealed tubes using 5% (w/v) solutions.

The pH of gelatin solutions shifts slightly during degradation (Ames, 1947) towards the isoelectric pH, the shift becoming very large at high pH values. This effect is minimized when sealed tubes are used, but no attempt was made to maintain the pH values constant during degradation with buffers since this was likely to introduce unwanted complications. All pH measurements were made with a Marconi pH meter (Marconi Instruments, London) using

a Doran 'Alkacid' glass electrode (L.S.B. Components Ltd., Stroud, Glos.) and a saturated calomel electrode (Cambridge Instrument Co. Ltd., London). When heating is effected at the isoelectric point, no change in pH occurs, at least up to 100° during 5 days. Consequently, when gelatin solutions were to be degraded at constant pH, the isoelectric pH was chosen.

Wherever possible, boiling solvents were used to attain constant temperatures, viz., $CHCl_3$ (60°), benzene (80°), water (100°). Temperatures of 75 and 90° were maintained by a thermostatically controlled oven to within ±0·25°.

N-Terminal residue determinations. These were carried out essentially as described by Courts (1954). In the reaction between partially hydrolysed gelatin and 1:2:4-fluorodinitrobenzene the precipitation of dinitrophenylgelatin (DNP-gelatin) was not successfully attained simply by acidification, and magnesium sulphate (saturated at and added at 40°, 150 ml.) was employed. To avoid overloading the column during chromatographic separation of DNP-amino acids, the amount of DNP-gelatin taken for hydrolysis was decreased to 100–300 mg. for the more degraded materials.

Rigidity determinations. These were done at 10°, after 18 hr. maturing, by the method of Saunders & Ward (1954). Mr P. R. Saunders kindly undertook the measurements on the samples, values of which are given in Tables 2, 4 and 5.

Reduced viscosity. The reduced viscosity η_t is expressed as $1/c \ln (\eta_{rel.})$, where c is concentration (%, w/v) and $\eta_{rel.}$ is $\eta_{solution}/\eta_{solvent}$. The conditions chosen were c approx. 0·5 in M-NaCl at pH 9. In these conditions the reduced viscosity is almost independent of c, and does not change with small variations of pH and salt concentration. The measurements were made at 35° in a no. 1 Ostwald viscometer (British Standards Institution (1937) 188) and these were kindly undertaken by Dr G. Stainsby. Variations in reduced viscosity may be regarded as closely corresponding to variations in M_w, the weight-average molecular weight (cf. Pouradier & Venet, 1952).

RESULTS

Degradation as a function of pH. (pH range, 1–12; temperature, 75°; time, 24 hr.) The conditions of time and temperature were chosen to give a substantial change of end-group values without degrading the protein to small peptides. Table 1 shows the extent of pH shift during 24 hr. heating. A comparison of the degradation results is shown in Table 2 as residues/100 000 g. gelatin, and again in Table 3 indicating the *N*-terminal residues as a percentage of the number of residues which could theoretically occur as end groups. Certain physical properties of the degraded protein are shown in

Table 1. pH *shift of gelatin solutions during degradation*

pH initial	1·1	2·1	3·1	4·1	5·1	7·0	9·1	10·2	11·2	12·2
pH final	1·4	2·8	3·4	4·3	5·1	6·7	8·7	9·6	9·9	10·4

Table 2. *Liberation of* N*-terminal groups and physical behaviour on degradation of gelatin as a function of* pH

For conditions, see text. End groups are expressed as moles/100 000 g. gelatin. M_n is number-average molecular weight calculated from end groups. η_t is reduced viscosity.

pH	1	2	3	4	5	7	9	10	11	12	Undegraded
Glycine	18·54	11·68	10·28	2·90	1·30	1·25	1·45	2·48	7·76	14·07	0·95
Serine	2·41	0·67	0·31	0·33	0·22	0·21	0·20	0·31	1·11	2·03	0·29
Threonine	2·53	0·58	0·36	0·21	0·16	0·14	0·13	0·21	0·42	1·26	0·12
Alanine	2·11	2·63	0·16	0·98	0·17	0·07	0·21	0·25	0·43	1·82	0·14
Aspartic acid	4·14	4·41	0·88	0·46	0·22	0·19	0·13	0·13	0·23	0·52	0·08
Glutamic acid	1·23	0·54	0·50	0·27	0·13	0·09	0·12	0·17	0·46	0·52	0·14
Others	0·82	0·45	1·10	0·23	0·05	0·05	0·00	0·05	0·22	0·41	0·00
Total	31·78	20·98	13·59	5·38	2·25	2·00	2·24	3·61	10·63	20·63	1·73
M_n	3000	5000	7000	19 000	45 000	50 000	45 000	28 000	9000	5000	58 000
η_t (½%)	0·031	0·077	0·090	0·143	0·33	0·35	0·30	0·20	0·114	0·042	0·57
M_n/initial M_n (%)	5·5	8·2	13	32	77	87	77	48	16	8·4	—
η_t/initial η_t (%)	5·4	13·5	16	25	58	61	53	35	20	7·4	—
Rigidity × 10^{-3} (dynes/cm.²)	Did not set			1·7	—	31·4	16·7		Did not set		75·4

Table 3. N*-Terminal residues as % of total residues of each kind present in gelatin degraded at different* pH *values (Table 2)*

pH	1	2	3	10	11	12	Undegraded
Glycine	5·0	2·9	2·7	0·65	2·1	3·7	0·25
Serine	7·5	2·1	0·95	1·3	3·4	6·3	0·70
Threonine	11·8	2·9	1·8	1·1	2·1	6·3	0·56
Alanine	2·1	2·6	0·16	0·25	0·43	1·8	0·14
Aspartic acid	9·0	9·6	1·9	0·26	0·49	1·2	0·17
Glutamic acid	1·8	0·90	0·72	0·25	0·65	0·81	0·20

Table 4. *Degradation of gelatin as a function of temperature*

For conditions see text. End groups are expressed as moles/100000 g. gelatin. M_n is number-average molecular weight calc. from end groups. η_i is reduced viscosity.

N-terminal residues	60°	80°	90°	100°
Glycine	1·39	2·64	3·64	6·06
Serine	0·24	0·27	0·37	0·47
Threonine	0·12	0·20	0·32	0·33
Alanine	0·17	0·53	1·26	2·17
Aspartic acid	0·16	0·54	1·00	1·89
Glutamic acid	0·12	0·16	0·33	0·36
Others	0·00	0·08	0·38	0·54
Total	2·20	4·42	7·30	11·82
Mol.wt. (M_n)	45 500	22 600	13 700	8500
Reduced viscosity	0·36	0·19	0·14	0·10
M_n/initial M_n (%)	79	39	24	15
η_i/initial η_i (%)	64	35	25	18
Rigidity $\times 10^{-3}$ (dynes/cm.^{2})	46	1·5	Did not set	

Table 5. *Degradation of gelatin as a function of time*

For conditions see text. End groups are expressed as g. residues/100000 g. gelatin. M_n is number-average molecular weight calculated from end groups. η_i is reduced viscosity.

	pH 5·1						pH 4·3					
	16 hr.	1 day	2 days	3 days	4 days	5 days	16 hr.	1 day	2 days	3 days	4 days	5 days
Glycine	2·23	2·47	3·64	5·00	6·14	6·58	3·43	4·25	5·32	7·32	8·89	10·84
Serine	0·28	0·39	0·37	0·43	0·45	0·63	0·49	0·58	0·65	0·75	0·87	0·91
Threonine	0·16	0·24	0·32	0·36	0·42	0·43	0·24	0·19	0·23	0·23	0·46	0·73
Alanine	0·83	0·79	1·26	1·80	2·05	2·18	1·27	1·93	2·53	3·14	3·49	4·03
Aspartic acid	0·49	0·65	1·00	1·61	2·50	2·45	0·81	1·11	1·71	3·03	4·87	7·60
Glutamic acid	0·19	0·17	0·33	0·26	0·23	0·60	0·27	0·36	0·52	0·54	0·86	1·24
Others	0·21	0·18	0·38	0·40	0·33	0·95	0·46	0·48	0·62	0·78	0·50	0·92
Total	4·39	4·89	7·30	9·86	12·12	13·82	6·99	8·90	11·58	15·79	19·94	26·27
M_n	22 700	20 400	13 700	10 100	8300	7200	14 300	11 200	8600	6300	5000	3800
η_i	0·20	0·175	0·14	0·119	0·10	0·094	0·13	0·11	0·096	0·088	0·081	0·073
M_n/initial M_n (%)	40	35	24	17	14	12·5	24	19	14·5	11	8·5	6·5
η_i/initial η_i (%)	36	31	25	21	18	17	24	20·5	17·1	15·7	14·5	13·0
Rigidity $\times 10^{-3}$ (dynes/cm.^{2})	7·9	2·7	Did not set				Did not set					

Table 2 and may be related to the corresponding results obtained by the *N*-terminal residue method.

Degradation as a function of temperature. (Temperatures 60, 80, 90, 100°; time, 48 hr.; pH 5·1.) Results are shown in Table 4.

Degradation as a function of time. (Times 16 hr., 1, 2, 3, 4, 5 days; temperature, 90°; pH, (*a*) 5·1, (*b*) 4·1 (initial)—in this instance the pH rose to 4·3 during 16 hr. and remained constant during 5 days.) Results at both pH values are shown in Table 5.

Calculation of M_n. A gelatin sample showing a total of T moles of *N*-terminal residues/100000 g. will show 1 mole in $100000/T$ g. If it is assumed that one molecule carries one *N*-terminal residue, then it follows that $100000/T$ represents the molecular weight of the sample. It is unlikely that this sample will be homogeneous with respect to molecular distribution. Consequently $100000/T$ represents a number-average molecular weight value.

DISCUSSION

Although small changes in temperature and time of heating may have a marked effect on the rate of release of free α-amino groups, changes in rate are more readily obtained by varying the pH of solution. Fig. 1 shows a smooth relationship to exist between peptide-bond breaking and pH. The rate is minimal at pH 7. Beyond the range pH 5–9, degradation increases sharply so that at pH 4, the number-average molecular weight, M_n, is only one-third of its original value (assuming one α-amino group/molecule) while at pH 3 it is one-eighth.

Glycine is the most frequently found *N*-terminal residue, accounting for over half of the total. The extent of breakdown of peptide bonds involving the amino groups of the other amino acids depends on whether acid or alkaline conditions of hydrolysis are used. The sum total of amino groups of aspartic and

glutamic acid residues shows a slight predominance over that for serine and threonine residues when degradation occurs in the acid range. Above pH 7, serine and threonine become much more important than the dicarboxylic acids (Fig. 2).

The preparation of oxhide collagen by the method of Bowes & Kenten (1948) and of ox-bone collagen by the method of Eastoe & Eastoe (1954) may lead to slight degradation of the material. Examination of the end groups of these materials (Courts, unpublished) showed glycine as the main *N*-terminal residue together with small amounts of serine, threonine, aspartic acid and glutamic acid. There is no indication, however, as to the order of release of these residues at this stage. Further hydrolysis of the collagens adds alanine to the list of *N*-terminal residues giving a qualitative correspondence with those present in gelatin. When gelatin is degraded, the next *N*-terminal residues to appear after the six given above are valine and phenylalanine respectively. Partridge & Davis (1950) have observed in studies of the acid hydrolysis of certain proteins (including gelatin) that aspartic acid is preferentially released in the free state and that this is often followed by preferential release of glutamic acid. An extension of this work with gelatin in these laboratories has shown that free amino acids are released in the order aspartic acid, glutamic acid, glycine, serine and alanine (serine was possibly contaminated with threonine). These are the same residues which occur as the principal *N*-terminal groups. It is reasonable to assume, therefore, that these residues are released as end groups as an intermediate stage before the complete separation as free amino acids. It is also very likely that the order in which they are detected in the free amino acid state is the same as the order (of descending magnitude) in which they are determined as *N*-terminal groups.

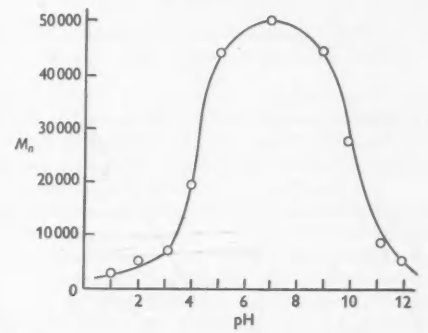

Fig. 1. Number-average mol.wt. of gelatin (M_n) after heating in solution for 24 hr. at 75° at different pH values. (M_n initially 58000.)

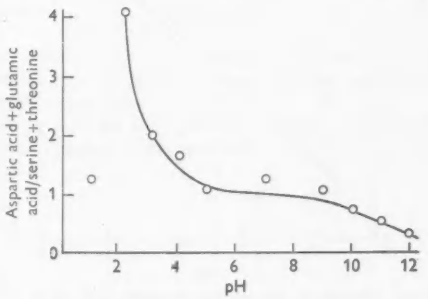

Fig. 2. The ratio of dicarboxylic amino acids to hydroxyamino acids released as *N*-terminal residues after heating gelatin at different pH values for 24 hr. at 75°.

The lability of certain peptide bonds has been studied by Levene, Synge and others using dipeptide hydrolysis. This and related work has been recently reviewed by Sanger (1952). Peptides so far examined involving glycine and alanine residues are relatively unstable irrespective of whether these are linked through —CO— or —NH— groups; glycylglycine is particularly unstable. Valyl peptides, on the other hand, appear to have a marked stability. Peptide bonds involving proline residues were found to exhibit unusual stability by Consden, Gordon, Martin & Synge (1947). Certain characteristics of gelatin degradation are in accord with this evidence. The ease with which gelatin is hydrolysed, for example, might be due in some measure to the large proportion of glycine residues (26·9 %) and the small proportions of valine and (leucine + isoleucine) residues (3·3 and 5·2 %). The predominance of glycine as an *N*-terminal residue would support this view. In a protein containing such a large proportion of residues of proline (14·8 %) and hydroxyproline (14·5 %), it might be expected that these amino acids would appear as end groups. Their non-appearance as *N*-terminal residues supports the view of Consden *et al.* (1947) of the great stability of peptide bonds involving proline. It is interesting that of the several peptides which have been isolated from gelatin partial hydrolysates (Synge, 1943; Gordon, Martin & Synge, 1943; Heyns, Anders & Becker, 1951; Schroeder, Honnen & Green, 1953) only one peptide is definitely identified as carrying hydroxyproline as the *N*-terminal residue while few appear to terminate with a proline residue. Smith (1953) has drawn attention to the ease with which many compounds of proline and hydroxyproline cyclize to form diketopiperazines. It is unlikely that similar cyclization occurs in polypeptides or proteins, although the possibility cannot be excluded. In the same way, Le Quesne & Young (1950) have shown that aqueous solutions of α-glutamyl peptides readily cyclize, probably into

stable pyrrolidone derivatives, but no such formation has been reported for α-glutamyl polypeptides.

The rate of breakdown of peptide bonds with temperature is obtained from Table 4. The temperature dependence of the rate of hydrolysis (k) is expressed by Arrhenius's equation

$$\frac{\mathrm{d}\ln k}{\mathrm{d}T}=\frac{A}{RT^2}$$

where A is the activation energy, R the gas constant and T the absolute-temperature. Under the present circumstances, the rate of hydrolysis may be replaced by the change in the number of end groups in a given time (r) measured in any consistent units, so that

$$\frac{\mathrm{d}\ln r}{\mathrm{d}T}=\frac{A}{RT^2}.$$

On integration this becomes

$$\ln r=-\frac{A}{RT}+\text{constant},$$

A, therefore, may be calculated from the slope of a graph of $\log_{10} r$ against the reciprocal of T. The plot of $\log_{10} r$ against $1/T$ is shown in Fig. 3 and the slope is given by $A/4{\cdot}61$. The value obtained for the activation energy of gelatin hydrolysis, 18 960 cal./mole is in fair agreement with certain previous results (Table 6).

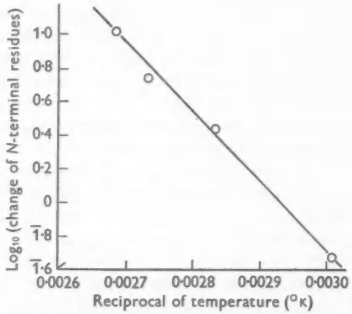

Fig. 3. The relationship between $\log_{10}$ (change of N-terminal residues) and the reciprocal of absolute temperature, the slope of which gives the activation energy of gelatin hydrolysis at pH 5·1.

Table 6. *Activation energies for hydrolysis of peptide bonds in gelatin (involving* —NH— *groups of specified residues)*

	Present work	Sheppard & Houck (1932)	Calc. from Greenberg & Burk (1927)
All bonds	18 960	19 920* – 26 540	2 480†
Glycine	15 360	—	—
Aspartic acid	22 670	—	—
Alanine	26 320	—	—
Serine and threonine	13 400	—	—

* By viscosity. † By amino nitrogen.

A plot of the change of total N-terminal residues with time (Fig. 4) shows an initial rapid increase which may be assumed to be due to the most labile peptide bonds. Once this stage of breakdown has been reached, hydrolysis proceeds at a steady rate. A somewhat similar general picture is reported by Pouradier & Venet (1952) from observations on intrinsic-viscosity changes. These workers consider, in particular, that by the time the molecular weight becomes about 7000 the remaining bonds are more resistant than those already broken, but there is no evidence of such a limit from N-terminal residue data.

It can be seen from Table 3 that the N-terminal residues developed correspond in no instance to exhaustion of the residues available, but it is possible that, when account is taken of the C-terminal residues involved, particular bonds may be found to have been exhausted.

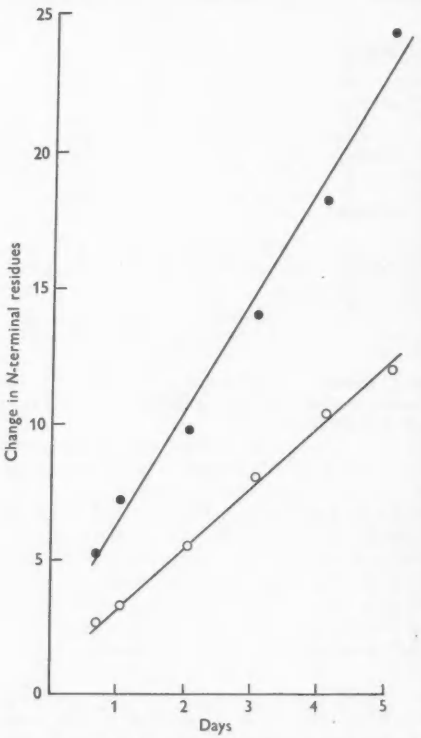

Fig. 4. Change in N-terminal residues with time after heating gelatin in solution at 90° and at constant pH. ●, pH 4·3; ○, pH 5·1. Residues as moles/1 000 000 g. gelatin.

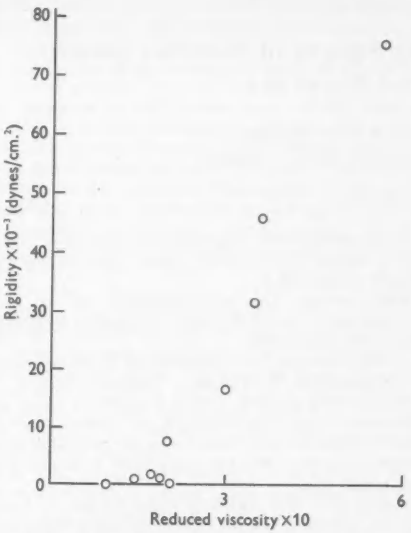

Fig. 5. The relationship between rigidity of gelatin gels and the reduced viscosity of the corresponding solutions.

The extent of degradation assessed by determination of *N*-terminal residues has been expressed as the percentage ratio $M_n/(M_n$ initial) (Tables 2, 4, 5) and compared with the results obtained by reduced viscosity, $\eta_i/(\eta_i$ initial). This is effectively a comparison between change in the number-average and weight-average molecular weights, and shows the type of relationship which might be expected.

If the results for rigidity are plotted against relative viscosity (Fig. 5) a consistent curve is not obtained. This is not surprising since it has already been established (Stainsby, Saunders & Ward, 1954) that the variation in rigidity is not solely related to changes in molecular weight.

SUMMARY

1. The *N*-terminal residues formed during degradation of gelatin under various conditions have been studied.

2. It is shown that the relative lability of different types of peptide bond varies with the pH of degradation.

3. The mean activation energy for peptide-bond hydrolysis at pH 5·1 is calculated as 18 960 cal./mole.

4. The extent of degradation, as measured by *N*-terminal residues, is compared with values obtained viscometrically. This is effectively a comparison between changes in number-average and weight-average molecular weights.

5. The kinetics of peptide-bond hydrolysis are discussed in relation to certain previous work.

I am grateful to Mr A. G. Ward and Dr G. Stainsby for the benefit of helpful criticism. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

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Hexosamine-containing Peptides in Spores of *Bacillus subtilis*, *B. megatherium* and *B. cereus*

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In a previous paper (Powell & Strange, 1953) the isolation of non-dialysable peptides containing hexosamine from exudates produced by germinating spores of *Bacillus subtilis* and *B. megatherium* was described. More precise chemical definition can now be given to these peptides. In the following report, a comparison is made between their constitution and that of the peptides present in extracts from disintegrated resting spores, and from vegetative cells. A strain of *B. cereus* isolated from air is also included in the investigation.

ORGANISMS AND METHODS

Preparation of germination exudates and cell extracts

The non-dialysable peptide produced by germinating spores of *B. megatherium* was studied in greatest detail. Well-washed resting spore suspensions of this organism were prepared and allowed to germinate spontaneously after heat activation as previously described. The peptide represented 10–15% of the solid exudate (Powell & Strange, 1953). Germination exudates from *B. subtilis* were obtained by incubating resting spores with L-alanine and glucose (Powell & Strange, 1953). Spores of a laboratory strain of *B. cereus* were obtained after growth for 48 hr. at 37° in potato-extract medium (Robinow, 1951), enriched with 1/10 vol. of casein hydrolysate and yeast extract (Gladstone & Fildes, 1940). The cultures were grown in shaken 1 l. flasks containing 200 ml. of medium. The spores of this strain of *B. cereus*, like *B. anthracis* (Hills, 1949), needed L-alanine, tyrosine, adenosine and glucose for germination. This requirement could be simplified by heating the resting suspension for 1 hr. at 60°, after which, adenosine and glucose were adequate for germination (Powell & Hunter, unpublished). Exudates were therefore prepared by incubating heat activated suspensions containing 2×10^9 spores/ml. in 1 mM adenosine and 5 mM glucose buffered with 30 mM phosphate pH 7·3. Germination exudates were filtered through sintered glass and freeze-dried before extraction and dialysis of the peptide fraction.

Vegetative cells of *B. megatherium* and of *B. cereus* were obtained by growth for 12 hr. on the sporulation media. They were washed 3 times with 0·9% NaCl and suspended in water.

Extracts from resting spores and from vegetative cells were obtained by disintegrating aqueous suspensions containing about 2×10^{10} cells/ml. in the Mickle (1948) tissue disintegrator at 4°, with Ballotini beads, size 12. The disintegrated suspensions were centrifuged at high speed, and the clear extracts freeze-dried.

Paper electrophoresis. The technique of Flynn & de Mayo (1951) was used in the buffer system described by Holt, Voigt & Gaede (1952).

Total nitrogen. This was determined by the micro-Kjeldahl method using the catalyst suggested by Chibnall, Rees & Williams (1943).

α-Amino nitrogen. This was estimated by the technique of Van Slyke, Dillon, MacFadyen & Hamilton (1941).

Paper chromatography. For the detection of amino acids and amino sugars, the technique of Consden, Gordon & Martin (1944) was used, followed by the method of Housewright & Thorne (1950) for quantitative determination of each amino acid.

Hexosamine. This was determined by the methods of Elson & Morgan (1933) with the modification suggested by Immers & Vasseur (1950), of Dische & Borenfreund (1950), and of Tracey (1952).

Acetyl. The *p*-toluenesulphonic acid method was used, hydrolysing at 110° for 18 hr. in sealed tubes, followed by steam distillation.

Carbohydrate. Total carbohydrate (excluding hexosamine) was determined by the method of Sørensen & Haugaard (1933).

Phosphorus, uronic acids and *pentose* were determined by the methods of King (1932), Dische (1947) and Meijbaum (1939).

Isolation of non-dialysable peptides from germination exudates and cell extracts

The freeze-dried germination exudate from *B. megatherium* which represented about 30% of the spore dry weight was extracted repeatedly with small volumes of water. A solution containing amino acids, peptide, small amounts of protein and calcium dipicolinate was thus obtained the relatively insoluble residue being practically pure calcium dipicolinate (Powell, 1953). The solution was dialysed at 4° for 48 hr., with continuous flow. Batches (1 g.) of germination exudate derived from $3·5 \times 10^{12}$ spores yielded 100–150 mg. of non-dialysable solid consisting almost entirely of the hexosamine-containing peptide. On one occasion during dialysis in two separate sacs the peptide was almost completely lost (see below). Some loss into the diffusate occurred when the dialysis time was extended to 4–5 days. It seemed, therefore, that the molecules might be barely of sufficient size to be retained by cellophan and, assuming a straight chain configuration, that the molecular weight might lie in the region of 10000 (see Addendum). On treatment of the solution of non-dialysable material with 2·5% trichloroacetic acid (TCA), a protein fraction was precipitated constituting about 4% of the non-dialysable solids. It gave a positive biuret reaction, and paper chromatography after acid hydrolysis showed most of the

common amino acids to be present. Ultraviolet absorption measurements indicated the absence of nucleic acids.

The solid material extracted from disintegrated resting *B. megatherium* spores represented about 50%, and from vegetative cells 45% of the cell dry wt. Freeze-dried spore and vegetative cell extracts were extracted with water and dialysed at 4°. The non-diffusible material and diffusate were concentrated *in vacuo*, and the diffusate was freeze-dried. The non-diffusible material was treated with 1/10 vol. of 25% TCA and centrifuged after standing for 30 min. The precipitated protein was washed with 2·5% TCA, dissolved in 0·1 M-$NaHCO_3$, dialysed and freeze-dried. The TCA solution and washings were combined and neutralized with solid $NaHCO_3$ then dialysed and freeze-dried. In these extracts, the TCA precipitate was found to be mainly nucleoprotein. The TCA-soluble fraction from resting spores contained a peptide very similar to that found in the germination exudate and constituting about 20% of the total extracted solids. From the acid-soluble fraction of *B. megatherium* vegetative cell extracts, a small amount of material was isolated, representing about 4% of the total extracted solids, and very different from the spore peptides in composition (see below).

Spore extracts and germination exudates from *B. cereus* and *B. subtilis* were treated in the same way, and TCA-soluble, non-dialysable fractions isolated. A 12 hr. culture of *B. cereus* which was already showing signs of sporulation, but contained no free spores was also examined.

Ethanol fractionation of non-dialysable peptides from Bacillus megatherium

The non-dialysable peptide fraction from germination exudates of *B. megatherium* could be partly precipitated from aqueous solution with 2 vol. of ethanol (Powell & Strange, 1953) but it was found, as is usual with this method of fractionation, that the results depended on the concentrations of peptide, ethanol and added electrolyte.

193 mg. of exudate peptide were dissolved in 5 ml. of water. Four drops of potassium acetate solution saturated at 20°, followed by 10 ml. (2 vol.) ethanol were added. A precipitate appeared, and the mixture was left to stand at 2° for 16 hr. The precipitate was centrifuged, dissolved in water, freed from salt by dialysis at 2°, and freeze-dried. A further 5 ml. of ethanol (3 vol.) was added to the ethanol–water mixture and the precipitate thus obtained, after standing, was treated as before. The material remaining in solution was freed from ethanol *in vacuo*, dialysed, and freeze-dried. Three fractions were thus obtained, the total yield being 171·2 mg. The fractions precipitated by 2 and 3 vol. of ethanol represented 41·3 and 35·0% respectively of the total. The fraction remaining in solution accounted for 23·7%.

An aqueous solution of *B. megatherium* spore extract peptide of the same strength (4%, w/v) was similarly treated with ethanol. It was found that 45% of the preparation was precipitated by the addition of 1 vol., and a further 45% by 2 vol. of ethanol. The remaining 10% was recovered from solution.

RESULTS

Bacillus megatherium *peptides*

The unfractionated non-dialysable peptides isolated from spore extracts and germination exudates of *B. megatherium* were found to be practically identical in composition (Table 1). After acid hydrolysis, followed by paper chromatography (Consden *et al.* 1944) each was shown to contain glutamic acid, alanine, αε-diaminopimelic acid, glucosamine, and an unidentified compound reacting with ninhydrin, the Elson & Morgan reagent (Partridge, 1948) and ammoniacal silver nitrate. These constituents were identified by comparing their positions and ninhydrin colour reactions with those of authentic compounds. Diaminopimelic acid was distinguished from cystine by its stability to hydrogen peroxide (Work, 1951) and its characteristic greyish purple colour with ninhydrin in chloroform-containing collidine (Woiwood, 1949). Eluted from a collidine chromatogram, it contained no phosphorus, and on paper ionophoresis (Wade & Morgan, 1954) it was distinguishable from ethanolamine phosphate. Using as solvent *tert*-butanol, 6 N-HCl and water in the volume ratio 70:1:29, it was unequivocally separated on paper from both cystine and ethanolamine phosphate and identified with αε-diaminopimelic acid.

It seems likely that the unidentified compound is a sugar amine. On two-dimensional chromatograms, using phenol–ammonia followed by collidine–lutidine, its position varied but was always different

Table 1. *Composition of non-dialysable peptides isolated from* B. megatherium (*g.*/100 *g.*)

Source of peptide	Sulphated ash	Total nitrogen	α-Amino nitrogen	Glutamic acid	Alanine	αε-Diaminopimelic acid
Germination exudate	5·8	7·37	2·91	5·3	9·0	5·1
Spore extract	7·0	7·35	2·75	5·1	8·1	5·6
Vegetative cell extract	—	7·7	—	*	—	—

Source of peptide	α-Amino nitrogen calculated from amino acid analyses	Hexosamine: Elson & Morgan	Hexosamine: Dische & Borenfreund	Hexosamine: Tracey	Acetyl	Total carbohydrate as glucose
Germination exude	2·72	40·3	42·3	56·2	10·9	1·24
Spore extract	2·69	43·2	43·5	57·2	12·0	5·0
Vegetative cell extract	—	6·58	—	—	—	16·3

* Chromatograms showed the presence of most of the common amino acids.

from that of glucosamine and chondrosamine. Using *tert*-butanol–HCl as solvent (Smith & Markham, 1950) batches of 30 mg. of hydrolysed peptide were chromatographed on Whatman no. 3 paper, and the unknown substance was eluted. On steam distillation in phosphate–borate buffer (Tracey, 1952) it yielded ammonia. It gave a reaction with Ehrlich reagent only after previous treatment with alkaline acetyl acetone. The resulting pigment showed maximum absorption between 510 and 520 mμ., the corresponding glucosamine derivative absorbing most strongly at 530–535 mμ. Although, at first, the colour given by the unknown closely resembled that given by glucosamine, it was less stable, and changed slowly to a salmon tint. At pH 9·5, the change of colour intensity produced with increasing concentrations of acetyl acetone (Immers & Vasseur, 1950) was very similar to that observed in the case of glucosamine (Fig. 1). The unknown substance also reacted with indole after deamination with nitrous acid (Dische & Borenfreund, 1950) to give a colour similar to that given by glucosamine but slightly more pink. The ratio of light absorption at 492 and 520 mμ. was approximately the same as with the glucosamine derivative. To simplify the presentation of data, the compound will be referred to as a hexosamine. A diagram of a typical two-dimensional chromatogram from hydrolysed peptide is shown in Fig. 2. The peptide had been freed from protein by TCA precipitation, which accounts for the absence of other amino acids found in the impure preparations of exudate peptide previously described (Powell & Strange, 1953).

The sugar amines were present as their acetyl derivatives and consisted mainly of glucosamine, together with smaller amounts of the unidentified amine sugar (see above). Hydrolysis of the peptides at several acid concentrations for varying periods followed by paper chromatography suggested that hydrolysis was complete after heating at 108° with 6N-HCl for 20 hr., but incomplete at lower acid concentrations and that in concentrated HCl, some destruction of the liberated hexosamine occurred. These indications were well supported by analytical results. Hydrolysis for 20 hr. at 108° with 0·5, 1, 3, 6 and 8N-HCl liberated respectively 24·4, 26·6, 33·1, 42·6 and 40·6% of hexosamine. Hydrolysis with 6N-HCl for 20 hr. was therefore adopted as standard procedure, although it seemed likely that even at this acid concentration, some decomposition of hexosamine occurred. Thus, glucosamine determinations on a standard solution of the hydrochloride using both the Elson & Morgan and the Van Slyke amino-nitrogen methods indicated a 11% destruction after heating for 20 hr. with 6N-HCl. The presence of the unidentified amino sugar giving a pigment with a different absorption maximum from that given by glucosamine in the Elson & Morgan reaction also reduced the accuracy of hexosamine determinations. Measurements were made of the colour with Ehrlich reagent at 520 and at 530 mμ. At 520 mμ., the values for hexosamine concentrations agreed well with those obtained by the Dische & Borenfreund method, while at 530 mμ. lower values were obtained, although this wavelength is nearer to the region of maximum ab-

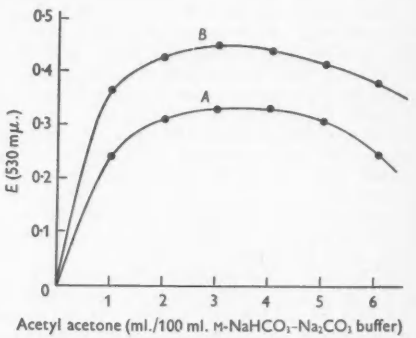

Fig. 1. The change of colour intensity with increasing concentrations of acetyl acetone in the Elson & Morgan reaction with *A* 40 μg. glucosamine and *B* unknown compound (suspected hexosamine).

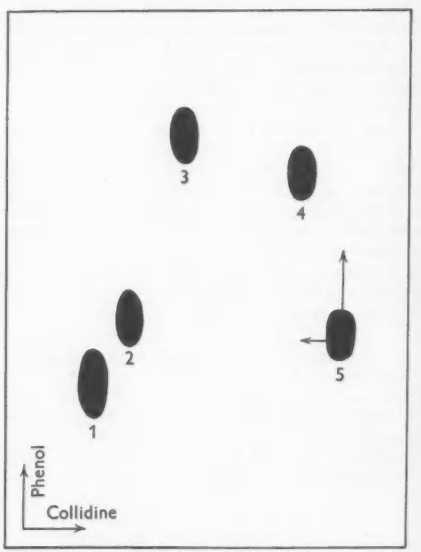

Fig. 2. Diagram of two-dimensional chromatogram of hydrolysed non-dialysable peptide from *B. megatherium* spores, developed with ninhydrin. 1, $\alpha\varepsilon$-diaminopimelic acid; 2, glutamic acid; 3, alanine; 4, glucosamine; 5, unidentified amine sugar.

sorption for glucosamine. It is therefore clear that the figures presented for hexosamine content of the peptides can be only approximate. Using the Tracey alkaline distillation technique, considerably higher values for hexosamine content were obtained. A typical set of results is given in Table 1. It may be significant that if the value for hexosamine nitrogen obtained by the Tracey method is added to that for α-amino nitrogen, all the nitrogen of the peptide is accounted for. If, on the other hand, the value obtained by the Elson & Morgan method is taken, there remains about 15% of the nitrogen unaccounted for.

The three amino acids present were estimated and found to occur in the ratio alanine (3), αε-diaminopimelic acid (1) and glutamic acid (1) to 8 of acetyl hexosamine.

The peptides from *B. megatherium* spores contained 0·03% of phosphorus and 0·2% of pentose. No uronic acid, purines or pyrimidines could be detected. The ash content, determined as sulphate, was 5–7% and consisted mainly of calcium.

The non-dialysable, TCA-soluble extract from vegetative *B. megatherium* cells contained only 6·58% of hexosamine (Table 1). Hydrolysis followed by paper chromatography showed that most of the common amino acids were present. The material was obviously different from the spore peptides and was obtained in very much smaller yield.

The peptides isolated from resting spores and from germination exudates were examined by paper electrophoresis (Flynn & de Mayo, 1951) with sodium acetate, acetic acid, sodium barbiturate buffer pH 8·5 (Holt *et al.* 1952). Both types of material appeared to contain a single component reacting weakly with naphthalene black and occupying corresponding positions on the paper. When a preparation of the exudate peptide which had not been treated with TCA was examined, the small protein fraction appeared as a very slow moving, strongly staining component well separated from the weakly staining peptide.

Analysis of ethanol-fractionated peptides from *B. megatherium* spores showed that, within the limits of the techniques used, the fractions from exudate peptides were identical. Each contained about 1% carbohydrate and we have not determined whether this is a constituent of the peptide molecule or an impurity derived from cell polysaccharides. The unfractionated extract peptide contained 5% carbohydrate. The carbohydrate content of the material precipitated by 1 and 2 vol. of ethanol was 5·4 and 4·2% respectively. The fraction remaining in solution contained 1·7%. All three fractions had very similar nitrogen and hexosamine contents, i.e. 7·0 ± 2%, and 40·7 ± 2%.

Viscosity measurements on aqueous solutions of the *B. megatherium* exudate and extract peptides gave interesting results. A microviscometer of the usual type was used giving a flow time of 141 sec. with water. In 1·33% (w/v) solution, the unfractionated exudate peptide gave a flow time of 190 sec., the fractions precpitated by 2 and 3 vol. of ethanol 204 and 192 sec., and the ethanol-soluble fraction 164 sec. The results on the extract peptide was less complete due to shortage of material. Here, 0·82% (w/v) solutions of the fractions precipitated by 1 and 2 vol. of ethanol gave flow times of 325 and 211 sec.

Bacillus cereus *and* Bacillus subtilis *peptides*

Very similar results were obtained with *B. cereus* and *B. subtilis*. Paper chromatograms on hydrolysates showed that the spore peptides isolated all contained alanine, αε-diaminopimelic acid, glutamic acid, glucosamine and the same unidentified compound suspected to be a sugar amine. Although the hexosamine content varied between 36 and 48% in these preparations, their general composition was remarkably similar. Analyses are summarized in Table 2. The reducing power of the hydrolysate is derived from the hexosamine liberated by 6N-HCl. Under these conditions, sugars are decomposed: hence the low value for reducing power of the *B. cereus* vegetative cell extract which actually contained a high proportion of polysaccharide. The reducing power is expressed in terms of glucose content. It may be significant that these values agree with the sugar amine content expressed as hexosamine, thus supporting though not confirming the suggestion that the unidentified compound is a hexosamine. No systematic study was made on the effect of acid concentration on liberation of hexosamine, but in a single experiment with *B. subtilis* spore extract peptide, 24·0% hexosamine was liberated during 20 hr. heating with 0·5N-HCl and 37·7% with 6N-HCl.

Table 2. *Composition of non-dialysable peptides isolated from* B. subtilis *and* B. cereus (*g.*/100 *g.*)

Source of material	Sulphated ash	Total nitrogen	α-Amino nitrogen	Hexosamine (Elson & Morgan)	Acetyl	Reducing power of hydrolysate (as glucose)	Total carbohydrate
B. subtilis spore extract	6·8	7·3	3·1	38·0	9·9	36·1	5·7
B. subtilis spore exudate	8·7	6·6	2·6	37·0	10·4	37·5	4·4
B. cereus spore extract	3·2	7·3	2·5	48·2	10·1	51·7	2·1
B. cereus spore exudate	2·7	7·3	2·9	42·5	9·9	50·0	4·7
B. cereus vegetative extract	3·1	4·9	3·2	8·9	3·9	8·9	51·6
							6·2

The *B. cereus* spore peptides appeared also to contain traces of other amino acids. These were reduced, but not entirely eliminated by treating the preparations with chloroform and amyl alcohol, a method used by Sevag (1934) to remove small amounts of protein. All the peptides isolated contained calcium. It is impossible to determine, at this stage, whether this is a constituent of the molecule or a contaminant. The non-dialysable peptide fraction from vegetative cell extracts of *B. cereus* contained most of the common amino acids, less than 10% of hexosamine, and 52% of polysaccharide determined as glucose (Sørensen & Haugaard, 1933). It is interesting that the vegetative culture used was already in the first stages of sporulation, most of the cells showing a round, slightly swollen, non-staining area.

Paper electrophoresis on the peptides from *B. subtilis* spores showed the presence of a single component reacting with naphthalene black. This occupied the same position on the paper as the corresponding derivatives from *B. megatherium* spore peptides. The material isolated from *B. cereus* spore extracts and exudates appeared to contain two components, one identical with that just described, and the other travelling much more slowly. When 10 mg. of peptide was separated into two fractions in this way, and then eluted from the paper, the yield of the slower moving fraction was 1·3 mg. and of the faster component 4·7 mg. The hexosamine content of the two fractions was 31·7 and 35·4% respectively, and each contained glutamic acid, alanine, $\alpha\epsilon$-diaminopimelic acid, glucosamine and the unidentified sugar amine.

DISCUSSION

Hexosamine-containing peptides of very similar and characteristic constitution have been isolated from extracts of resting spores and from germination exudates of *B. subtilis*, *B. megatherium*, and *B. cereus*. Peptides of this type were absent or possibly present in only very small amounts in extracts from a young vegetative culture of *B. megatherium* and from vegetative cells of *B. cereus* which were in the first stages of sporulation. It therefore appears that the peptides may be built up at a comparatively late stage of the sporulation process.

All the spore peptide preparations contained alanine, glutamic acid, $\alpha\epsilon$-diaminopimelic acid and the acetyl derivatives of glucosamine and of another suspected but unidentified hexosamine. The *B. cereus* spore peptides contained small amounts of the other common amino acids which may have been derived from protein or from other types of non-dialysable peptide. Paper electrophoresis indicated the presence of one component in the peptides from *B. megatherium* and *B. subtilis*, and of two components of very similar hexosamine and amino acid content in the peptides from *B. cereus*. Ethanol fractionation of the *B. megatherium* peptides gave products of the same chemical composition, although in the case of the extract peptide, the carbohydrate content of the fraction soluble in 2 vol. of ethanol, was considerably lower than that of the precipitated fraction. It seems possible that the peptide preparations contain molecules of different chain lengths and that during germination some depolymerization of the peptide may occur. This view is supported by the viscosity measurements. In the experiment mentioned early in this paper where there was an almost complete loss of exudate peptide through the dialysis sacs, a greater degree of depolymerization than usual may have occurred.

Hydrolysis of *B. megatherium* exudate peptide showed that approximately two-thirds of the sugar amine molecules were liberated by 0·5N-HCl, while the remaining one-third were much more tightly bound. Since the peptide contained an insignificant amount of phosphorus, this finding does not support the suggestion of Bendich & Chargaff (1946) that ease of liberation of amine sugars is associated with phosphorus-free, and difficulty with phosphorus-containing polymers.

We are unable to attach any biochemical significance to the spore peptides beyond the suggestion previously made (Powell & Strange, 1953) that they may be derived from the spore surface when the permeability of this structure is modified during germination, or damaged mechanically.

SUMMARY

1. Non-dialysable peptides of very similar constitution have been isolated from testing spore extracts and from germination exudates of *B. megatherium*, *B. subtilis* and *B. cereus*. These peptides are not present in appreciable quantities in extracts from vegetative cells.

2. The non-dialysable spore peptides contained $\alpha\epsilon$-diaminopimelic acid, glutamic acid, alanine, and the acetyl derivatives of glucosamine and an unidentified sugar amine.

We wish to thank Dr R. B. Record for freeze-drying facilities, Mr F. Belton for growing large spore crops, Dr C. E. Dent for a specimen of ethanolamine phosphate and Mr F. A. Dark and Mr A. G. Ness for assistance with the analyses. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

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ADDENDUM

Physico-Chemical Properties and Molecular Weight of Spore Peptides from *Bacillus megatherium*

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Specimens of the exudate peptide and of the extract peptide prepared from *Bacillus megatherium* according to the above procedure have been examined in the electrophoresis apparatus and the ultracentrifuge. The diffusion constant and partial specific volume have also been determined and these have been combined with the sedimentation constant to give a figure for the molecular weight of the peptide. The questions of homogeneity, monodispersity and molecular shape are also briefly considered.

METHODS

Electrophoresis. This was carried out at 0° in the medium size (10 ml.) U-tube of the Tiselius electrophoresis apparatus and observations were made with the diagonal Schlieren optical system. The solutions were made up to 1% from the freeze-dried sample and were dialysed for 2 days against the buffer used.

Ultracentrifuge. Observations were made in the Spinco analytical ultracentrifuge fitted with the diagonal Schlieren optical system. Solutions were made up directly by dissolving a weighed amount of the freeze-dried material in the buffer used. The rotor speed was 59 780 rev./min. in all experiments and exposures were taken every 64 min.

Diffusion. The diffusion of the samples was followed in the electrophoresis apparatus using the standard 10 ml. U-tube. The solutions, approx. 1%, were dialysed for 2 days against the buffer used. The measurements were made at 0°.

Buffer. All the work was carried out in a sodium acetate (0·1M), NaCl (0·1M) buffer adjusted to pH 4·5 with acetic acid.

RESULTS

Spore exudate peptide

The specimen examined consisted of the 2·5% trichloroacetic acid soluble fraction, which had been dialysed and freeze-dried but had not been subjected to any ethanol fractionation.

Electrophoresis. A current of 9 mA was passed for 4 hr. through a 1·2% solution. The schlieren pattern showed only a single component, migrating anodically. The small stationary boundary clearly evident on the anode limb but hardly perceptible on the cathode side was assumed to be due to the usual buffer gradient. The mobility calculated from the movement of the cathode (descending) boundary was $0{\cdot}38 \times 10^{-4}$ cm.^{2}/sec./v.

Sedimentation. The boundary in the ultracentrifuge also showed the presence of only one component having a low rate of sedimentation of about 1 Svedberg. Fig. 1 shows the pattern obtained with a 2% solution. As seen from the results in Table 1 (all the S_{20} values have been reduced to sedimentation in water and are given in Svedberg units) the sedimentation constant is somewhat concentration dependent and the relationship appears to be linear over the range examined.

Determinations of S become unreliable at concentrations much below 1% owing to the rapid spread of the sedimenting boundary and linearity has been assumed in extrapolating the S_{20} values to

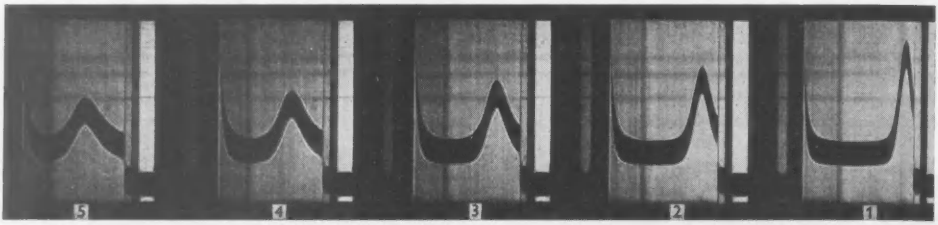

Fig. 1. Sedimentation pattern of the exudate peptide. 2% Concentration in sodium acetate (0·1 M) NaCl (0·1 M) buffer pH 4·5. Speed 59780 rev./min. Interval between exposures = 64 min. Sedimentation from right to left.

Table 1. *Sedimentation constants of the exudate peptide*

Concentration, %	2·50	2·00	1·50	1·00
S_{20} (Svedbergs)	0·89	0·98	1·06	1·12

Table 2. *Diffusion constants from sedimentation diagrams*

Concentration, %	2·50	2·00	1·50	1·00
Mean $D_{20} \times 10^7$	3·4	4·3	4·8	5·8

zero concentration. The value so obtained S_{20} $(c \to 0) = 1{\cdot}28$ has been used in calculating the molecular weight.

Diffusion. Since a diffusion experiment carried out on a 1% solution showed a very nearly symmetrical concentration distribution, it appeared that diffusion was not appreciably concentration dependent at concentrations below 1%, and the values obtained in this experiment have been used in calculating the molecular weight. The height (H) and area method of calculation was used. Nine exposures were taken over a period of 30 hr. and $1/H^2$ was plotted against time for each of the two boundaries in the U-tube. The points fell very closely on a straight line cutting the time axis at a value of -6×10^3 sec. and this point was taken as zero time in calculating the diffusion constant. The average value obtained for D at 0° was $2{\cdot}904 \times 10^{-7}$ and this was corrected to diffusion in water at 20° by the formula

$$D_{20} = D \text{ buffer at } 0^\circ \times \left(\frac{\eta_0}{\eta_{20}}\right)_{H_2O} \times \left(\frac{\eta_{\text{buffer}}}{\eta_{H_2O}}\right) \frac{293}{273}$$

to give a value of $5{\cdot}88 \times 10^{-7}$ for D_{20}.

The partial specific volume of the material was determined in buffer as 0·656 for a 1·3% solution and 0·654 for a concentration of 0·6%. The measurements were made by the pycnometric method at 25°.

Taking a mean value for V of 0·655 together with $S_2 = 1{\cdot}28$ and $D = 5{\cdot}88 \times 10^{-7}$ we obtain mol. wt. = 15300.

The frictional ratio, f/f_0, has a value of 2·4, corresponding to an axial ratio of 30 in the case of a rod-shaped unhydrated molecule. So large a degree of molecular asymmetry is perhaps surprising in the case of a low molecular weight peptide.

The degree of polydispersity. Both in electrophoresis and in ultracentrifuging the peptide shows only a single moving boundary. By calculating 'diffusion constants' from the sedimentation diagrams and comparing the values obtained with the true diffusion constant we have endeavoured to obtain information on the degree of polydispersity of the material. The problem is complicated by the dependence of sedimentation on concentration which leads to an artificial sharpening of the sedimenting boundary, an effect which becomes more marked the higher the concentration. The sedimentation diagrams were analysed by the height and area method taking times from the point at which the rotor reached full speed. A correction term $(1 - \omega^2 st)$ was applied to each exposure to allow for spread due to variation in the centrifugal field with distance from the rotor centre (see Svedberg & Pedersen, 1940, p. 277). No marked drift was found for the D values during a run. The mean values corrected to water at 20° are given in Table 2. By linear extrapolation to zero concentration, the diffusion coefficient calculated from the boundary spread during sedimentation is about $7{\cdot}1 \times 10^{-7}$, a figure not greatly higher than the true diffusion constant. Making allowance for the various errors inherent in the method, especially in the case of a substance with a low sedimentation and a rather high diffusion constant, it may be concluded that the material is either monodisperse or polydisperse only to a very limited extent (compare, for example, the gross polydispersity found in certain polysaccharides, e.g. glycogen (Record, 1948), where spreading coefficients of 30–100 times the true diffusion constant are found).

Spore extract peptide

This material, extracted from freeze-dried *B. megatherium* spores, consisted of the 2·5% trichloroacetic acid soluble fraction, dialysed and freeze-dried as in the case of the exudate peptide.

Table 3. *Sedimentation constants of the extract peptide*

Concentration, %	2·00	1·50	1·00
S_{20} (Svedbergs)	0·93	1·01	1·13

Electrophoresis. The material was examined under precisely the same conditions as the exudate peptide and practically identical behaviour was observed. Electrophoretically it showed only a single component with a mobility, calculated for the cathode limb, of $0{\cdot}42 \times 10^{-4}$ cm.^{2}/sec./v in close agreement with that found for the exudate peptide.

Sedimentation. Here also the results show a close parallel with those obtained for the exudate peptide. The corrected S_{20} values are given in Table 3, and the extrapolated value of $S_{20}(c \to 0) = 1{\cdot}32$. At the same concentration, the rate of spread of the sedimenting boundary appeared to be slightly less than in the case of the exudate peptide.

Diffusion. An anomaly was observed here in the form of a sharp peak surmounting the main concentration gradient curve and displaced slightly to the buffer side of the main peak. The picture suggests the presence of a small percentage of a high molecular contaminant, though there is no evidence of this in the ultracentrifuge diagrams. The presence of this contaminant makes the analysis of the diffusion curves difficult. The main part of the sample, however, diffuses at a rate very near to that found for the exudate peptide. Without further purification it is doubtful whether a more detailed examination of the extract peptide sample is justified.

DISCUSSION

As seen in the main part of this paper, the crude non-dialysable fraction of the exudate yielded 96 % of a 2·5 % trichloroacetic acid soluble fraction, this constituting the sample examined here, whereas the crude extract dialysate gave only a 20 % yield when similarly treated. It appears that the germinating spores allow only material of relatively low molecular weight, the exudate peptide, to pass into solution. The extract peptide, on the other hand, has been separated by a single fractionation from the entire spore contents and it is perhaps not surprising that it does not reach the same standard of homogeneity and monodispersity as the exudate peptide.

Evidence of some polydispersity has been adduced in both samples by viscosity measurements on fractions separated by precipitation at various ethanol concentrations. The differences were much more pronounced in the extract than in the exudate peptide, thus supporting the conclusions already reached.

SUMMARY

1. The exudate peptide from germinating spores of *B. megatherium* appears to be homogeneous in electrophoresis and in sedimentation at pH 4·5.

2. At 20°, it has a sedimentation constant at zero concentration of $1{\cdot}28 \times 10^{-13}$, a diffusion coefficient of $5{\cdot}88 \times 10^{-7}$ and a partial specific volume of 0·655. These data give a figure of 15300 for the molecular weight and 2·4 for the frictional ratio, pointing to marked molecular asymmetry.

3. The material is essentially monodisperse.

4. A peptide extracted from resting spores of *B. megatherium* appears to be physico-chemically identical to the exudate peptide except for the presence of a small amount of contaminant.

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The Biological Action of Substances Related to Thyroxine

9. THE EFFECT OF BUTYL 4-HYDROXY-3:5-DIIODOBENZOATE ON THE DEIODINATION OF TRIIODOTHYRONINE IN THE RAT

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(*Received* 17 *March* 1954)

The metabolic interactions of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB) with thyroxine and triiodothyronine in intact rats and mice have been previously reported (Sheahan, Wilkinson & Maclagan, 1951; Maclagan, Sprott & Wilkinson, 1952; Maclagan & Wilkinson, 1952; Sprott, Wilkinson & Maclagan, 1953). These studies have led us to advance the hypothesis that BHDB exerts its effects by inhibiting the deiodination both of thyroxine and of triiodothyronine *in vivo*. Evidence in support of the first part of this theory has been obtained by studying the effect of BHDB on the urinary iodide of rats treated with thyroxine labelled in the 3′ and 5′ positions with ^{131}I (Wilkinson & Maclagan, 1953*a*; Wilkinson, Sprott, Bowden & Maclagan, 1954). The present communication

reports a similar study with BHDB and triiodothyronine, the latter labelled with ^{131}I in the 3′ position.

Preliminary reports of some of the results described in this paper have appeared elsewhere (Wilkinson & Maclagan, 1953*b*, 1954).

EXPERIMENTAL

Preparation of ^{131}I*-labelled* 3:5:3′*-triiodo-*L*-thyronine*

Various methods for the preparation of the labelled triiodothyronine were studied. At the suggestion of Professor A. Wormall, the isotope-exchange method (Miller, Anderson, Madison & Salley, 1944; Frieden, Lipsett & Winzler, 1948) was tried, but, though good yields of material of high specific activity were obtained, it was decided not to use this product since we could not be certain that exchange had taken place exclusively in the 3′ position. A method based upon that used by Clayton, Free, Page, Somers & Woollett (1950) for the preparation of labelled thyroxine was finally employed, the product being purified via the hydrochloride by a procedure similar to that used by Gross & Pitt-Rivers (1953).

Isotope-exchange method. Of the several procedures studied the following gave the best results. Triiodothyronine (10 mg.) was suspended in a mixture of 0·1 N acetate buffer of pH 5·3 (5 ml.) and *n*-butanol (1 ml.) containing $Na^{131}I$ (50 μc). The mixture was shaken at intervals while being heated under reflux on a steam bath for 42 hr. The butanol was removed by evaporation at 20°/1 mm. and the residue diluted to approx. 8 ml. The solid was collected and washed with water until no radioactivity could be demonstrated in the washings (about 10 of 3–4 ml. each were required). The product was then dried over silica gel at 20°. After making allowance for radioactive decay, the total utilization was 90%. The product was shown by paper chromatography to have undergone no decomposition. When the butanol was omitted, the utilization was only 65% after similar treatment. The uptake was negligible when the mixture was shaken mechanically for 42 hr. at 20°, irrespective of the presence or absence of the solvent.

Iodination of 3:5-*diiodo-*L*-thyronine.* A solution of KI (9·6 mg., 0·058 m-equiv.) and $Na^{131}I$ (5 mc) in water (2 ml.) was acidified with 2 N-HCl (0·15 ml.) and covered with ether (5 ml.). The mixture was treated with 0·1 N-H_2O_2 (1·3 ml., 0·13 m-equiv.), added over 20 min., and shaken at intervals for 4 hr. The ether layer was then transferred slowly to a solution of 3:5-diiodo-L-thyronine (15·3 mg., 0·029 m-equiv.) in 50% aqueous ethylamine (0·5 ml.). The iodination required about 2 hr., towards the end of which, water (2 ml.) was also added. The ether was removed in a current of air, and the mixture evaporated to approx. 0·5 ml. at 30°/1 mm. to remove excess ethylamine. The residue was transferred to a centrifuge tube, about 5 ml. of water being added, and the solution heated to 60°. The pH was adjusted to 4–5 with acetic acid to precipitate the product. After several hours at 0–5°, the solid was collected and washed 3 times with water (10 ml.). The crude product was dissolved in 2 N-HCl (5 ml.) at 70–80°, the solution was filtered, and the filtrate, while still hot, adjusted to pH 4–5 with NaOH and acetic acid. It was then stored at 0–5° for 2 hr., after which the crystalline deposit was separated in a centrifuge and washed 3 times with water (10 ml.). The product was again dissolved in 2 N-HCl and the crystallization procedure repeated.

The 3:5:3′-triiodo-L-thyronine thereby obtained in 80% yield was shown by paper chromatography to be free from thyroxine and iodide. The chromatograms were developed by the ceric sulphate–arsenious acid method of Bowden & Maclagan (1954) and also by autoradiography on Ilford Industrial B X-ray film. The only possible contaminant not detectable by this procedure is diiodothyronine. The extent of contamination with this substance must have been small since the product had m.p. 231–232° (decomp.). Gross & Pitt-Rivers (1953) report 233–234° (decomp.). As diiodothyronine has no known physiological activity and could not be radioactive, the product was considered suitable for use in the experiments described in this paper. It had a specific activity of 61·5 μc/mg., which after allowing for radioactive decay, corresponds to 44% of the theoretical utilization of radio-iodide.

For use in the animal experiments, a solution of 100 μg./ml. in 50% (v/v) aqueous propylene glycol was prepared, the pH being adjusted to 8–9 with diethanolamine. Immediately before using this solution paper chromatograms were run to ensure the absence of interfering substances. For administration to rats, the concentrated solution was diluted with physiological NaCl solution to produce the required dose in 1 ml.

Solutions of radioactive triiodothyronine appeared to be much more stable than those of labelled thyroxine (Wilkinson *et al.* 1954), for after storage for several weeks no trace of iodide could be detected chromatographically, using either the ceric sulphate–arsenious acid method or autoradiography.

Animal experiments

In each experiment male rats, each within ±15 g. of the mean weight, which in different experiments varied from 140 to 200 g., were fed on cubed stock diet (Thomson, 1936) and tap water, both *ad lib.* Groups of three similarly treated animals were kept in metabolism cages, from which the urine and faeces were collected daily. After suitable treatment (Wilkinson *et al.* 1954) the radioactivity of the excreta was determined by counting in a liquid counter (Veall, 1948) connected to a standard scaler.

The solution of triiodothyronine was administered by subcutaneous injection. Suspensions of BHDB (50 mg./ml.) in 1% aqueous methoxycarbonylcellulose were given orally, the controls receiving only the medium by the same route.

RESULTS

The results obtained in a typical experiment in which doses of 6 μg./rat of triiodothyronine were given are shown in Fig. 1. As in earlier experiments half of the rats received BHDB (50 mg./rat) on the day before the triiodothyronine injection and 25 mg./rat on each succeeding day throughout the experiment. It has been established that the urinary radioactivity after the administration of labelled iodothyronine derivatives consists almost exclusively of iodide whilst that in the faeces is almost entirely due to thyronines (Gross & Leblond,

1951*a*, *b*; Roche, Michel & Tata, 1952; Wilkinson & Maclagan, 1953*a*). Consequently any differences between the urinary radioactivities of different groups must be a measure of variations in iodide output. It is clear from Fig. 1 that there is a marked reduction in the urinary iodide when BHDB is given along with triiodothyronine. Similar results were obtained when doses of 30 μg. triiodothyronine/rat were given (Table 1).

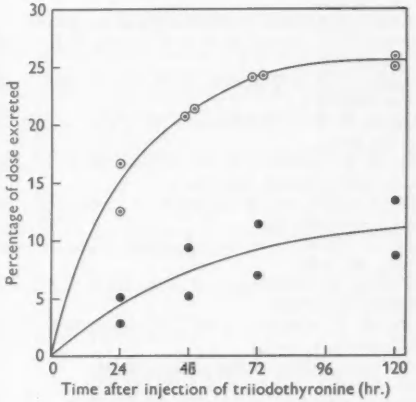

Fig. 1. The effect of BHDB upon the urinary radioactivity of rats treated with a single subcutaneous injection of ^{131}I-labelled triiodothyronine. ⊙, the combined output of a group of three rats treated with triiodothyronine (6 μg./rat); ●, the combined output of a group of three similarly treated rats given in addition daily oral doses of BHDB (25 mg./rat).

Table 1. *The effect of BHDB on the urinary radioactivity of rats treated with* ^{131}I*-labelled triiodothyronine*

Each row of figures refers to the combined output of a group of three rats. See text for dosage of BHDB.

Dose of triiodothyronine (μg./rat)	BHDB	Percentage of dose excreted after 24 hr.	48 hr.	120 hr.
30	Absent	11·0	18·4	20·6
		23·2	31·6	34·0
30	Present	2·8	6·3	9·0
		3·5	8·0	10·5
50	Absent	14·2	18·3	20·2
		20·3	25·8	28·2
		20·6	25·2	27·8
		19·2	23·7	26·8
50	Present	5·5	10·1	14·6
		3·3	5·9	8·3
		2·5	6·6	9·3
		5·4	*	*

* The metabolism cage containing this group of rats was damaged during the second day of the experiment and the urine became contaminated with faeces.

In order to exclude the possibility that the results were due to chance individual variations in the animals used in the experiment, the rats were kept for 5 weeks after which no radioactivity could be detected in the urine. Another experiment using 50 μg. doses of triiodothyronine was then performed. In this case rats previously given BHDB + triiodothyronine were given triiodothyronine alone, and those which originally had the hormone alone now received both drugs. The results which appear in Table 1 show a depression of urinary iodide similar to those obtained in the first experiments.

In all three experiments the diminished urinary iodide loss in the group treated with BHDB was balanced by an increased faecal radioactivity due to thyronine derivatives (Maclagan & Wilkinson, 1954). There was thus no significant difference between the total excretion of labelled compounds in the two groups.

DISCUSSION

The suggestion that BHDB might owe its metabolic activity to interference with processes of deiodination was made by Maclagan *et al.* (1952) to explain the difference in its effects on mice treated with triiodothyronine and thyroxine. That it reduces the output of urinary iodide of rats injected with thyroxine was shown by Wilkinson & Maclagan (1953*a*) and Wilkinson *et al.* (1954). The present work completes this aspect of the study by showing that the output of urinary iodide is also reduced in similar experiments with triiodothyronine. Since BHDB has no effect upon the urinary excretion of injected iodide (Wilkinson *et al.* 1954), these reductions in iodide output must be a measure of the change in the degree of deiodination. The present observations, therefore, are in accord with our hypothesis, at least in intact rats. In thyroidectomized rats, however, Roche, Deltour & Michel (1953) have reported that BHDB increased the rate of deiodination of thyroxine labelled in the 3′:5′ positions with ^{131}I. We are not in a position to offer any explanation of this apparent contradiction, but it is conceivable that the metabolism of BHDB may differ in intact and thyroidectomized animals.

The possibility that the metabolic effects of BHDB might be due to alterations in the total rate of excretion of thyroxine or triiodothyronine has been considered, but is unlikely in view of our results. Thus in the experiments reported it was found that the lowered urinary excretion of iodide in the groups treated with BHDB was balanced by a higher faecal excretion of thyronine derivatives, so that the overall rate was similar in both groups. It

follows that the action of BHDB in inhibiting the effect of thyroxine on the oxygen consumption of mice and its opposite action in the case of triiodothyronine (Maclagan *et al.* 1952) cannot have been due to changes in the total excretion rate. They must therefore reflect alterations in the activity of the circulating thyroid hormone, probably consequent upon inhibition of deiodination processes.

SUMMARY

1. Details are given for a method of preparation from 3:5-diiodo-L-thyronine of triiodothyronine labelled in the 3′ position with ^{131}I. Radioactive triiodothyronine appears to be much more stable in solution at pH 8–9 than labelled thyroxine.

2. The labelled compound was injected into rats, with or without simultaneous administration of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB), and the radioactivity of the excreta was measured.

3. Administration of BHDB caused a marked reduction in the output of urinary iodide in intact rats after a single injection of labelled triiodothyronine at a number of different dose levels. This is interpreted as being due to interference with the deiodination of this compound.

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Studies in Carotenogenesis

13. THE CAROTENOIDS OF THE FLOWER PETALS OF *CALENDULA OFFICINALIS*

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The presence of β- and γ-carotenes, lycopene, violaxanthin and rubixanthin in *Calendula officinalis* (pot marigold) has already been reported in the early investigations of Zechmeister & Cholnoky (1932) and Kuhn & Grundmann (1934); lycopene was absent from the very yellow varieties (Zechmeister & Cholnoky, 1932). Preliminary tests on the petals of a variety of *C. officinalis* producing very rich dark orange flowers showed that they contained a complex mixture of carotenoids including some which had not previously been reported. The investigation was therefore continued in greater detail.

EXPERIMENTAL

Materials. Fully opened flowers were gathered from a garden in north-west Cheshire. The seeds were obtained from Messrs Elsoms, Ltd., Spalding.

Extraction of pigments. The flower petals were removed from the calyx, placed in a Waring Blendor, covered with acetone and blended for 2 min. Most of the acetone extract was decanted, and the remainder removed from the residue by filtration through a sintered glass funnel. The residue was then returned to the Blendor and extracted again with acetone. This procedure was repeated until all the pigments had been extracted. The acetone extracts were combined and an equal volume of ether was added, followed by water

(slowly) until two layers formed. The carotenoids passed quantitatively into the ether layer and the water-soluble pigments (flavones, etc.) remained in the lower layer, which was discarded. The ether layer was washed twice with small quantities of water to remove acetone, dried (Na_2SO_4), filtered, and the solvent removed *in vacuo* at room temperature. The residue was dissolved in light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) and subjected to chromatographic analysis.

Chromatography and identification of pigments

The pigments were separated by column chromatography on either activated alumina (grade '0', P. Spence and Co., Widnes), deactivated alumina made by treating grade '0' with methanol (Goodwin & Srisukh, 1949), or by an appropriate mixture of the two.

The well authenticated carotenoids were identified (*a*) by their relative positions on the column when chromatographed under the standardized conditions used in this laboratory, and (*b*) by the shape and location of their absorption spectra (Goodwin, 1952*a*, *b*, 1953, and unpublished observations). These two criteria can generally give unambiguous identification; cases where any possible ambiguity exists are emphasized in discussing the results obtained.

Two main chromatographic separations of the carotenoids of *C. officinalis* were carried out; the first on deactivated alumina (separation 1) and the second on a mixture of 3 parts activated and 2 parts deactivated alumina (separation 2). The resolution obtained on the first chromatogram is given in Table 1. Fraction 1 which ran straight through the column of weakened alumina and consisted of a mixture of hydrocarbons was resolved on the more active alumina, resulting in the separation of the components listed in Table 2.

Table 1. *First separation of* C. officinalis *carotenoids*

Adsorbent, deactivated alumina; developer light petroleum containing varying amounts of ether; zones numbered in order of increasing adsorptive power.

Zone	Colour	Absorption maxima in light petroleum (mμ.)	Concn. (%, v/v) of ether required to remove zone from column	Provisional identification
1	Yellow-brown	—	0 (Moved straight down column)	Mixture of carotenes
2	Lemon-yellow	418, 438, 467	2·5–5	?
3	Lemon	~415, 434, 463	5	? *cis* Isomer of 2
4	Lemon	401, 420, 446, 466	10	Mixture of 3 and 5
5	Lemon	~401, 420, 446	12	Flavochrome
6	Brown-orange	459, ~482	15	Rubixanthin-like
7	Lemon	410, 422, 448	15–20	Mutatochrome
8	Lemon	400, 422, 424	25	Mixture containing mostly 7
9	Brown	423, 446, 472	50	Lutein
10	Lemon	400, 422, 440	50–60	Flavoxanthin + chrysanthemaxanthin
11	Lemon	400, 422, 446	75	
12	Brown	402, 422, 443	Ether	—
13	Khaki (small)	Indeterminate band	Ether + 1% ethanol	Oxidation product
14	Khaki (small)	420, 441, 460	Ether + 5% ethanol	? Neoxanthin

Table 2. *Resolution of fraction* 1 (*Table* 1) *into its component polyenes*

Adsorbent, 3:2 mixture of activated and deactivated alumina; developer, light petroleum containing varying amounts of ether; zones lettered in order of increasing adsorptive power.

Zone	Description	Absorption spectrum maxima in light petroleum (mμ.)	Concn. (%, v/v) of ether required to remove zone from column	Provisional identification
1*A*	Colourless; non-fluorescent in ultraviolet light	Indeterminate	0	Possible traces of phytoene
1*B*	Colourless; vivid green fluorescence in ultraviolet light. Tendency to separate into two zones	330, 348, 367	1	Mixture of phytofluene isomers
1*C*	Brownish yellow	~425, 449, 474	2–3	β-Carotene
1*D*	Lemon-yellow	380, 400, 422, 451	5	Mixture of ζ-carotene and pigment *X* (Goodwin & Osman, 1953)
1*E*	Brownish	~410, 432, 456, 483	10	Mixture
1*F*	Pink	438, 462, 487	10–15	Mixture
1*G*	Yellow	439, 467, 485	15–20	?
1*H*	Pink	439, 468	20	? Lycopene isomer
1*J*	Bright pink	443, 469, 495	25–30	Neolycopene A
1*K*	Brick red	445, 471, 499	30	Lycopene

Table 3. *Resolution of mixed fractions* IE, IF, IG (*Table* 2) *into their components*

Adsorbent, fully activated alumina; developer, light petroleum containing varying amounts of ether; zones lettered in order of increasing adsorptive power.

Zone	Description	Absorption spectrum maxima in light petroleum (mμ.)	Concn. (%, v/v) of ether required to remove zone from column	Provisional identification
EA	Pinkish-brown	430, 458, 487	35	γ-Carotene
1*EB*	Pink	443, 469, 495	50–60	Neolycopene A
1*EC*	Bright pink	446, 471, 500	60–70	Lycopene

Examination of the fractions

Fractions 1A–1M. The following were identified after purification by rechromatography: β-carotene (1*C*), neolycopene A (1*J*) and lycopene (1*K*). Fraction (1*B*) was rechromatographed on active alumina using light petroleum containing 10% (v/v) ether as developer and separated into two colourless zones which were detected by their green fluorescence in ultraviolet light. Both the zones had the same absorption spectrum ($\lambda_{max.}$ 330, 348, 367 mμ.), characteristic of phytofluene. The lower zone represents phytofluene which is a *cis*-polyene (Petracek & Zechmeister, 1952) and the upper zone *trans*-phytofluene.

Fraction 1*D* was chromatographed on fully activated alumina and on elution with light petroleum separated into two zones: 1*DA*, the less strongly adsorbed pigment with absorption maxima at 405, 424, 451 mμ. and 1*DB* (λ_{max} 381, 401, 425 mμ.); 1*DA* is the pigment *X* described by Goodwin & Osman (1953) and 1*DB* is the ζ-carotene of Porter & Zscheile (1946).

Fractions 1*E*, 1*F* and 1*G*, which originally were not well separated, were combined and rechromatographed on activated alumina (see Table 3). The main pigment is γ-carotene, accompanied by small amounts of lycopene and neolycopene.

Fraction 1*H* was rechromatographed on strong alumina but was essentially homogeneous, only traces of neolycopene being present. It is probably prolycopene I (Zechmeister & Pinckard, 1947). Fraction 1*J* was rechromatographed and separated into lycopene and neolycopene, whilst fraction 1*K* was almost entirely lycopene.

Fractions 2–8 (*xanthophylls containing one polar grouping*). It appears from their adsorptive power that fractions 2–8 are carotenoids containing one oxygen atom as a hydroxyl, epoxy or keto group. This is confirmed by the fact that none of these pigments is hypophasic in the partition test with 90% (v/v) aqueous methanol.

Fraction 2 from its chromatographic behaviour and the shape and position of its absorption spectrum, is a pigment not previously described; fraction 3 appears to be a *cis* isomer of fraction 2 because its spectrum has the same shape but the maxima have been shifted slightly to a lower wavelength. Similarly, fraction 5, characterized by a spectrum very similar to that of ζ-carotene, but adsorbed above instead of below lycopene, is flavochrome (Karrer & Jucker, 1950; Goodwin, 1952*c*).

Fraction 6 has the adsorption properties and the position of its main absorption band very similar to those of rubixanthin (Kuhn & Grundmann, 1934); its subsidiary bands were, however merely inflexions and not true bands, whilst those of rubixanthin are well marked and indistinguishable from that of the parent γ-carotene (Mackinney, 1935; Goodwin, unpublished observations).

Fraction 7 has properties similar to those of flavochrome but with its absorption bands at slightly higher wavelengths and with a slightly greater adsorptive power than flavochrome; it is probably mutatochrome.

Fraction 8 was a mixture containing mostly fraction 7 (mutatochrome), together with small amounts of a pigment with at least one absorption band at a lower wavelength than the lowest of mutatochrome (402 mμ.). Persistent efforts to purify this fraction were not successful, but the spectrum of the best fraction obtained strongly suggested that the pure pigment would probably be aurochrome.

Fractions 9–14 (*xanthophylls containing more than one oxygen grouping*). Fraction 9 was identified in the usual way as lutein; Fractions 10–12 spread on the column and do not separate cleanly. They have the characteristic adsorption properties and absorption spectrum of flavoxanthin and chrysanthemaxanthin. These pigments are extremely difficult to separate (Karrer & Jucker, 1950) and from the spread of these fractions one can only conclude that both pigments are probably present. Fraction 13 is a small band with an indeterminate spectrum and appears to be a carotenoid degradation product, whilst the final zone (14), although not pure, exhibited a spectrum which suggests that it might contain small amounts of neoxanthin.

Quantitative determination of pigments. The amounts of each pigment present were determined by measuring the *E* (at $\lambda_{max.}$) of each fraction dissolved in a known volume of light petroleum and comparing it with the known $E^{1\%}_{1\,cm.}$ values (at $\lambda_{max.}$) of the pure pigment (Goodwin, 1952*a–c*). For unknown pigments $E^{1\%}_{1\,cm.}$ ($\lambda_{max.}$) was assumed to be 2500. All carotenoids which have been obtained crystalline have $E^{1\%}_{1\,cm.}$ values varying between 1900 and 3100, most of the values being around 2500.

Spectroscopic measurements. These were carried out either on a Beckman Model DU quartz spectrophotometer or on the corresponding Unicam (SP. 500) instrument.

RESULTS

Polyene hydrocarbons

The following polyene hydrocarbons have been identified in the petals of *C. officinalis*: phytofluene, β-carotene, γ-carotene, pigment *X*, ζ-carotene, and lycopene. The phytofluene fraction (1*B*, Table 2) was resolved into two polyenes with very similar absorption spectra. The lower adsorbed fraction is phytofluene whilst that adsorbed above this fraction is *trans*-phytofluene. The recent work of Petracek & Zechmeister (1952) has shown that the normal naturally occurring phytofluene is probably a *cis* form; and it is very difficult to decide whether

trans-phytofluene occurs naturally or is an artifact, for it can be formed from *cis*-phytofluene by very gentle treatment (Petracek & Zechmeister, 1952). Similarly, about 30% of the lycopene isolated was in the form of a *cis* isomer, neolycopene A, and again this is so easily produced from the all-*trans* form that it is probably an artifact. On the other hand, Fraction 1*G* which is probably prolycopene I, (a poly-*cis*-lycopene) cannot be an artifact, for it is not possible to detect poly-*cis*-lycopenes in heat- or iodine-isomerized lycopene solutions (Zechmeister & Petracek, 1952).

This appears to be the first occasion on which pigment *X* has been observed naturally; it was first found in cultures of *Phycomyces blakesleeanus* grown on media containing diphenylamine: it is not produced by this organism on normal media (Goodwin & Osman, 1953). It is separable from ζ-carotene only with difficulty; its absorption spectrum is recorded in Fig. 1.

Xanthophylls

The following xanthophylls have been identified: flavochrome, mutatochrome, lutein, and probably both flavoxanthin and chrysanthemaxanthin. Traces of aurochrome and neoxanthin are probably also present. Fraction 2 (Table 3) cannot be identified with any known pigment; its absorption spectrum is recorded in Fig. 1. Fraction 6, from the position of the absorption maximum of its main band and from its adsorptive power would appear to be rubixanthin (3-hydroxy-γ-carotene) which has previously been reported in the petals of *C. officinalis* (Kuhn & Grundmann, 1934). The essentially single-banded spectrum of fraction 6, however (Fig. 1), suggests a ketocarotenoid rather than a hydroxy-γ-carotene; the latter, as stated previously, has a well-defined three-banded spectrum, indistinguishable from that of γ-carotene. In this variety of *C. officinalis* it does appear that not rubixanthin but a closely related compound is produced.

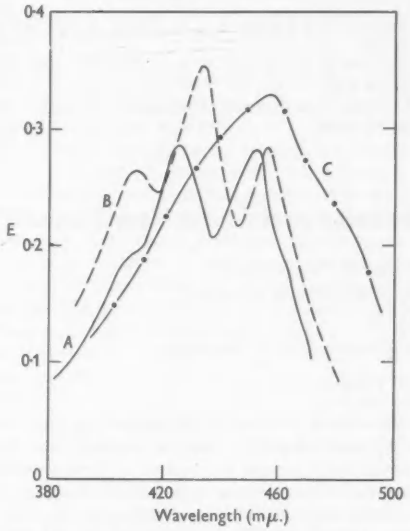

Fig. 1. Absorption spectra in light petroleum of *A*, pigment *X*, ——; *B*, unknown pigment (fraction 2, Table 1), – – –; *C*, rubixanthin-like pigment, •—•.

Quantitative experiments

A quantitative separation of the pigments into xanthophylls and carotenes (including traces of the colourless polyenes) showed that each group represented about one-half of the total pigments present. The relative amounts of the components in these two main fractions are given in Tables 4 and 5. Although

Table 4. *Relative amounts of hydrocarbon polyenes in* C. officinalis

Polyene hydrocarbons represent 45·3% of the total polyenes present.

Polyene	Wt. (as % of total polyene hydrocarbons present)
Phytofluene*	0·4
β-Carotene	13·8
Pigment *X*	2·2
ζ-Carotene	7·8
γ-Carotene	14·1
Unknown (zone 1*G*, Table 2)	2·6
Lycopene†	59·1

* A trace of *trans*-phytofluene was also present, but may be an artifact.

† The lycopene fraction includes 30·1% neolycopene A; this may also be an artifact.

Table 5. *Relative amounts of xanthophylls in* C. officinalis

Xanthophylls represent 54·7% of the total polyenes present.

Xanthophylls	Wt. (as % of total xanthophylls present)
Unknown (zone 2, Table 1)	5·2
(?) *cis*Isomer of unknown	1·9
Flavochrome	2·8
Rubixanthin-like	7·4
Mutatochrome	17·8
Aurochrome*	7·4
Lutein	5·2
Flavoxanthin†	24·5
Chrysanthemaxanthin†	26·3
Oxidation product	0·4
? Neoxanthin	0·9

* Never completely separated from mutatochrome; this represents an upper limit.

† Separation not complete; these values are only approximate.

there may be some variations in the relative amounts during the development of the petals, it is obvious that lycopene is the main carotene, and that flavoxanthin and chrysanthemaxanthin the main xanthophylls.

DISCUSSION

The examination of *C. officinalis* petals confirms two generalizations which have been made with regard to carotenoid distribution in petals (Goodwin, 1952*c*); (*a*) that 5:8-epoxides (flavochrome, mutatochrome, aurochrome, flavoxanthin and chrysanthemaxanthin) are widely distributed in petals in relatively large amounts and (*b*) that lutein, although present is not, as in green leaves, the major xanthophyll. Furthermore, the investigation adds emphasis to the recent observations made on berries (Goodwin, 1953, and unpublished observations) that when examined with the aid of present knowledge concerning separation and identification of trace carotenoids, the pigment mixture is found to be much more complex than was originally thought. This is mainly due to the fact that the pioneer investigators were mainly interested in isolating the major pigments in order to examine them chemically. The biological significance of these complex mixtures is at present quite obscure.

The present investigation confirms the original observation of Zechmeister & Cholnoky (1932) and Kuhn & Grundmann (1934) on the major pigments present, except that violaxanthin was not detected and although a 'rubixanthin-like' pigment was observed, rubixanthin itself was not detected. These differences may be varietal.

SUMMARY

1. The following hydrocarbon polyenes have been found in the petals of a 'dark' variety of *Calendula officinalis* (pot marigold): phytofluene, β-carotene, γ-carotene, ζ-carotene and lycopene. Pigment *X* closely associated with ζ-carotene, was also present. A poly-*cis*-lycopene was also present; it was probably prolycopene I.

2. The xanthophylls present were flavochrome, mutatochrome, lutein, flavoxanthin and chrysanthemaxanthin; traces of neoxanthin and aurochrome are also probably present. Two pigments were unidentified; pigment 6 was similar to, but not identical with rubixanthin.

3. The relative distribution of these pigments in the petals is recorded.

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Purification and some Properties of Cephalosporin N, a New Penicillin

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A species of *Cephalosporium* isolated by Brotzu (1948) from a sewage outfall off Sardinia can produce at least six different antibiotics (Burton & Abraham, 1951; Crawford *et al.* 1952). One of these substances, named cephalosporin N, has been reported to be a new type of penicillin, distinguished clearly from the penicillins previously encountered by its hydrophilic character and by an activity of the same order of magnitude against a number of Gram-positive and Gram-negative bacteria (Abraham, Newton, Crawford, Burton & Hale, 1953). An account of some of the biological properties of the substance has been given by Heatley & Florey (1953). This paper describes a process whereby cephalosporin N

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can be obtained from culture fluid of the *Cephalosporium* in a form which appears to be at least 75% pure. The production of the active culture fluid is described by Kelly & Miller (1954) and by Hale (1954). The chemical structure of the antibiotic is discussed in the following paper (Newton & Abraham, 1954).

Early experiments established that cephalosporin N was an acidic substance which was rapidly inactivated by dilute acid or alkali, by certain heavy metal ions, and by the enzyme penicillinase. The instability of the substance imposed limitations on the methods available for its purification which were similar to those encountered with the common penicillins. Unlike the latter, however, cephalosporin N could not be extracted from an aqueous solution by organic solvents such as amyl acetate, or butanol, and a different process was therefore required for removing it from the culture fluid.

The process of purification consisted of three main stages: adsorption on a column of charcoal (pH 6) and elution with aqueous acetone; chromatography on acid-washed alumina from aqueous acetone; and countercurrent distribution at 3° in a system composed of water, phenol, carbon tetrachloride and 2:4:6-trimethylpyridine sulphate at pH 6·1. Antibacterial activities were expressed in terms of an arbitrary unit which is described by Kelly & Miller (1954). Good yields were only obtained from charcoal and alumina columns if the amount of adsorbent was not greatly in excess of that required to remove all the active substance. Some irreversible changes occurred and became serious when the adsorbent was present in large excess. The displacement of certain components by others on the columns evidently played an important part in the separation that was achieved.

In a simple phenol–water system the partition coefficient of cephalosporin N was large enough for the convenient use of countercurrent distribution only at a pH lower than that at which the antibiotic was stable. Addition of 2:4:6-trimethylpyridine to the mixture, however, greatly increased the relative solubility of the antibiotic in the organic phase when the pH of the aqueous phase was 6·1. In the system used for countercurrent distribution 2:4:6-trimethylpyridine acted as both a carrier and a buffer, and it could finally be removed under conditions which did not cause inactivation of cephalosporin N. Such systems may well prove to be of value for the separation of other acidic hydrophilic substances.

Cephalosporin N was also subjected to countercurrent distribution in a system composed of ethanol, *sec.*-butanol and a concentrated aqueous solution of ammonium sulphate at pH 6·0, but this system was not used in preparative work, because of the subsequent difficulty of isolating the product in a salt-free form.

Many of the chemical properties of cephalosporin N were analogous to those of other penicillins. Chemical methods of assaying the latter could therefore be applied to the former. Assuming a value for the molecular weight of cephalosporin N these assays provided an estimate of the degree of purity of the preparations obtained.

RESULTS

Purification of cephalosporin N

Adsorption on and elution from charcoal. The active culture fluid was filtered and its pH adjusted to 6·0. It was then percolated down a column packed with granulated charcoal prepared and buffered at pH 6 as described in the Experimental section. Elution was carried out with 60% (v/v) acetone. The total amount of cephalosporin N eluted was 97% of that in the culture fluid. One fraction of the eluate contained 69% of the cephalosporin N in one-fifteenth of the volume of the culture fluid. The activity of the material in this fraction was 4 units/mg. Any cephalosporin P which was present in the culture fluid (Burton & Abraham, 1951) remained adsorbed on the charcoal, but could be removed by elution with an alkaline aqueous solution of acetone.

Chromatography on alumina. Column 1. Acetone was added to selected fractions of the eluate from the charcoal column until its concentration was 70% (v/v) and the pH of the resulting solution was adjusted to 6·0. A small amount of precipitate of low activity came out of solution and was discarded. The supernatant was percolated through a column of acid-washed alumina (pH 4·0), at 2°, until 80% of the alumina was saturated with cephalosporin N (see Experimental). Elution was begun with 20% (v/v) acetone, which removed inactive material, and then continued with 0·02N sodium hydroxide. The cephalosporin N was eluted in a broad band which contained 70% of the active material in one-fifth of the volume of the solution added to the column. The activities of the fractions, based on dry weights obtained by evaporating samples at 100°, varied from 18 to 33 units/mg. (see Experimental and Table 5).

Column 2. Acetone was added to fractions selected from the first alumina column (26·5 units/mg.) until its concentration was 20% (v/v). The pH of the solution was adjusted to 6·0 and it was then chromatographed on a second column of acid-washed alumina (pH 4·6) at 2°. The size of the column was such that 80% of the alumina was saturated with cephalosporin N. Elution was carried out with 0·01N sodium hydroxide and the eluate collected in small fractions. These fractions

contained 80 % of the cephalosporin N which had been adsorbed, with activities varying from 25–40 units/mg. (see Experimental and Table 6). The activities are based on dry weights obtained by evaporating samples at 100°.

Eluates from the second alumina column were combined in groups according to their activity. Acetone was removed and the aqueous solutions were freeze-dried. The following amounts of nearly white powders, containing mainly the sodium salt of cephalosporin N, were obtained from 240 l. of filtered culture fluid: 8·5 g. at 38 units/mg.; 2 g. at 37 units/mg.; 2·5 g. at 27 units/mg.; 1·4 g. at 25 units/mg. Attempts to raise the activity of this material by further chromatography on alumina were unsuccessful.

Table 1. *Recovery of cephalosporin N after chromatography on charcoal and alumina*

Fraction	Total units passed on to next stage	Proportion of activity of original filtrate (%)
Fermentation filtrate	2 060 000	100
From charcoal column	1 410 000	69
From alumina column 1	950 000	46
From alumina column 2	583 000	28
Solid (mainly sodium salt)	494 400	24

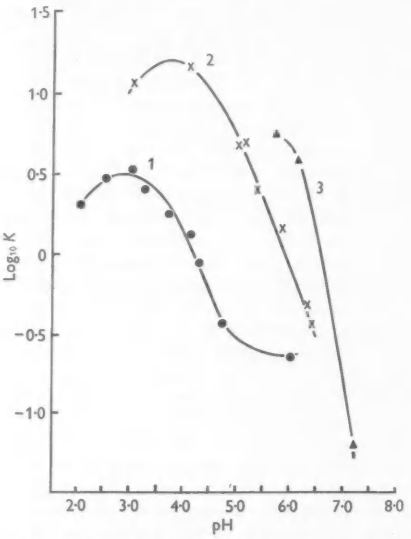

Fig. 1. Partition of cephalosporin N in phenol–water systems at various pH values. (1) Phenol–water. (2) Phenol–*NN*-dimethylaniline–water. (3) Phenol–carbon tetrachloride–2:4:6-trimethylpyridine–water (system 1). K = [activity in phenol phase]/[activity in aqueous phase].

Table 1 summarizes the amounts of cephalosporin N which were recovered at each stage of the purification process.

Partition coefficients in phenol–water systems. The partition coefficient K (activity in phenol phase/activity in aqueous phase) of cephalosporin N between phenol and water was found to vary with the pH of the aqueous phase in the manner shown in Fig. 1 (curve 1). The value of K reached a maximum at pH 3·0. When 12 % (v/v) of *NN*-dimethylaniline was added to the phenol phase of this solvent system, and the pH adjusted with sulphuric acid, K was greatly increased over the range pH 3·0–5·0 and now reached maximum at pH 4·0 (Fig. 1, curve 2). When the stronger base, 2:4:6-trimethylpyridine, was added, the maximum value of K was at about pH 5·3.

A solvent system suitable for countercurrent distributions with a mobile upper aqueous phase, and a ratio of upper phase to lower phase of 3 to 2 by volume, was obtained from the following mixture: phenol saturated with water (40 vol.), carbon tetrachloride (40 vol.), 2:4:6-trimethylpyridine (7·1 vol.), water saturated with phenol (350 vol.), 10N sulphuric acid (1 vol.). The pH of the aqueous phase (glass electrode) was 6·0–6·2. This was called system 1. Fig. 1 (curve 3) shows the change in K when the pH of this system was varied by the addition of different amounts of sulphuric acid.

Countercurrent distribution. System 1. In a preliminary experiment cephalosporin N (20 units/mg.) was purified by twenty-four fundamental transfers followed by 101 withdrawals of the aqueous phase from tube 24, in system 1, at room temperature. Material which was recovered as a barium salt from the active band in the withdrawn series had an activity of 38 units/mg. However, 35 % of the initial activity was unaccounted for, indicating that breakdown of cephalosporin N had occurred during the experiment. The product was therefore redistributed in system 1 at 3°. After fifteen fundamental transfers, sixty-five withdrawals of the aqueous phase were made from tube 15. Fig. 2 shows the antibacterial activity, ninhydrin colour density and dry weight of selected fractions. The results indicate that the active substance could account for 73 % of the preparation. The appearance of substances A and B confirmed that breakdown of cephalosporin N had taken place during the previous experiment. In the present experiment approximately 85 % of the antibacterial activity of the starting material was accounted for. The material in fractions 28–58 of the first withdrawn series (aqueous phase) was recovered as a barium salt and precipitated by acetone from aqueous solution. The activity of the product was 55 units/mg.

In another experiment a preparation of cephalosporin N obtained from the second alumina column (28 units/mg.) was distributed in system 1 at 3°. After sixteen fundamental transfers, eighty-one withdrawals of the aqueous phase were made from tube 16. Fractions 59–77 of the withdrawn series yielded a barium salt with an activity of 58 units/mg.

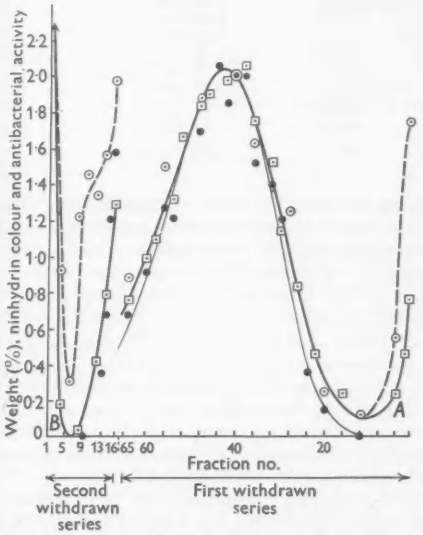

Fig. 2. 80-Transfer distribution of cephalosporin N in system 1 (phenol–carbon tetrachloride–2:4:6-trimethylpyridine–dilute sulphuric acid, pH 6·1). Ratio (upper phase/lower phase) 3:2, by vol. Upper phase mobile. ●—●, relative antibacterial activity; ——, calculated curve ($K = 0{\cdot}24$); ⊙—⊙, % of dry weight; ⊡—⊡, relative ninhydrin colour density.

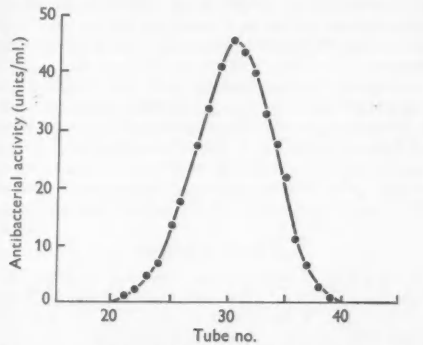

Fig. 3. 49-Transfer distribution of cephalosporin N in system 2 (ethanol–ammonium sulphate–sodium phosphate–citric acid–water, pH 6·0). Upper phase mobile. ●—●, antibacterial activity; ——, calculated curve ($K = 1{\cdot}633$).

Partition coefficients in alcohol–aqueous ammonium sulphate systems. In systems composed of ethanol, or 2-ethoxyethanol, and concentrated ammonium sulphate solutions at pH 5·1, cephalosporin N was appreciably soluble in the alcohol-rich phase. In the system ethanol–water–ammonium sulphate (2·8:6·5:3·0, by weight) the partition coefficient K (activity in alcohol phase/activity in aqueous phase) was 1·57. In the system: 2-ethoxyethanol–water–ammonium sulphate (2·3:5·6:2·0 by weight) K was 2·0. A solvent system suitable for countercurrent distribution of cephalosporin N was obtained from the following mixture: 0·1 M disodium hydrogen phosphate (50·5 ml.); 0·1 M citric acid (14·7 ml.); ammonium sulphate (30 g.); ethanol (30 ml.); *sec.*-butanol (5 ml.). This was called system 2. The pH of the aqueous phase (glass electrode) was 6·0.

Distribution in system 2. A preparation of cephalosporin N (15 units/mg.) was subjected to a forty-nine transfer (fundamental) distribution in system 2 at 16°. The distribution of antibacterial activity is shown in Fig. 3. There was no indication of the presence of more than one active component. No loss of antibacterial activity was detected.

Some properties of cephalosporin N

Cephalosporin N barium salt with an activity of 58 units/mg. was a white powder, readily soluble in water but sparingly soluble in methanol and insoluble in ethanol. It had $[\alpha]_D^{20} + 187°$ in water (c, 0·6). It was not precipitated from concentrated aqueous solution by *NN*-dibenzylethylenediamine, which forms a sparingly soluble salt with benzylpenicillin (Szabo, Edwards & Bruce, 1951). It contained C, H, N and S, but no halogen or phosphorus. After drying *in vacuo* at room temperature, elementary analysis showed: N, 8·7; S, 6·9%. The atomic ratio of N:S was therefore 3:1. The material showed a strong ninhydrin reaction, and electrometric titration revealed the presence of a basic group of pK 9·8, as well as acidic groups with pK values of 3·2 and less than 2·8. The equivalent weight of the barium salt, estimated from the span of the titration of the basic group, was 495. Assuming that the substance gave the same ninhydrin colour yield per gram molecule as leucine, the molecular weight of the barium salt was estimated to be 450.

Additional evidence for the presence of a basic group in the active molecule was provided by the change with pH of the partition coefficient between phenol and water (Fig. 1). On lowering the pH of the aqueous phase the partition coefficient rose to a maximum at about pH 3·0 and then fell, suggesting that the antibiotic reached an isoelectric point in this region.

Table 2. *Chemical estimations of the antibacterial activity of the pure sodium salt of cephalosporin N*

For methods see text.

Method	Activity of preparation (units/mg.)	Moles of cephalosporin N/381 g.	Estimated activity of pure sodium salt (units/mg.)
Carbon dioxide	13·6	0·181	75
	42·0	0·493	85
	58·0	0·795	73
Hydroxylamine	17·8	0·182	98
	28·0	0·342	82
Iodine	3·5	0·050	70
	17·8	0·238	75
	28·0	0·364	77
Penicillinase	32·0	0·387	82

Table 3. *Estimated relative activities of pure cephalosporin N and benzylpenicillin*

Organism	Activity of cephalosporin N / Activity of benzylpenicillin
Staphylococcus aureus, NCTC 6571	0·01
Staphylococcus aureus (penicillin resistant)	0·04
Pneumococcus, type 1	0·06
Streptococcus viridans, NCTC 3165	0·06
Vibrio cholerae	2
Salmonella typhi	3
Salmonella enteritidis	6
Klebsiella pneumoniae	6

Preparations of cephalosporin N, like benzylpenicillin, evolved carbon dioxide on acid hydrolysis, reacted with hydroxylamine to form a hydroxamic acid, absorbed iodine after alkaline inactivation, and underwent hydrolysis with the liberation of an acidic group on treatment with penicillinase. These reactions were used for the chemical assay of cephalosporin N in terms of benzylpenicillin. Carbon dioxide was determined as described by Abraham, Baker, Chain & Robinson (1949). The reaction with hydroxylamine was carried out by a modification of the method of Ford (1947) and the reaction with iodine by the method of Alicino (1946). The acid liberated on inactivation with penicillinase at pH 7·0 was determined by titration in a manner similar to that described by Wise & Twigg (1950). Assuming that one molecule of cephalosporin N behaved like one molecule of benzylpenicillin in these reactions, and that impurities were inert, the reactions could be used to calculate the number of gram molecules of cephalosporin N in a given weight of an impure preparation. The weight of cephalosporin N in such preparations was then calculated on the assumption that the molecular weight of its barium salt was 427 and its sodium salt 381 (see Newton & Abraham, 1954). This provided an estimate of the degree of purity of the preparations and of the antibacterial activity of the pure substance. The results are shown in Table 2. The values indicate that the antibacterial activity of cephalosporin N sodium salt is about 80 units/mg. and therefore that the preparation of the barium salt obtained in the present work was about 80 % pure. On this basis the data of Heatley & Florey (1953) have been used to estimate the relative activities of pure cephalosporin N and benzylpenicillin against a number of Gram-positive and Gram-negative bacteria (Table 3).

The properties of cephalosporin N described in this paper suggest that it is a penicillin carrying an aminodicarboxylic acid residue in the side chain. A structure of this type would account for the strongly hydrophilic nature of the molecule.

EXPERIMENTAL

Antibacterial assay. Solutions were assayed by the method of Brownlee *et al.* (1948), using *Klebsiella pneumoniae* (Departmental strain) as a test organism, or by a similar method using *Salmonella typhi*, strain 'Mrs S.' (Felix & Pitt, 1935).

Filtration of culture fluid. The culture fluid (280 l.) was cooled to 20° by circulating cold water through the jacket of the fermenter. The pH of the culture fluid (8·0) was adjusted to 6·0 by adding 1 l. of 11 N-HCl. The fluid was then pumped through a cloth bag filter and the filtrate was passed through a filter press containing Fords Sterimats (F.C.B. size PA/20 cm.). The final filtrate (240 l.) was perfectly clear.

Charcoal column

Preparation of the charcoal. Granular charcoal (20 to 60-mesh/in., 270 kg., grade BO, manufactured by Farnell Carbons Ltd., London) was suspended in tap water and transferred to a glass column made from two 3 ft. sections of 9 in. industrial glass pipe. The dimensions of the packed charcoal were 23 × 120 cm. About 30–50 l. of N-HCl were percolated down the column until the effluent had a strongly acid reaction. The evening before use the column was washed with 50 l. of distilled water and then with phosphate

Table 4. *Charcoal column for adsorption of cephalosporin N from culture fluid*

For details see text.

Fraction	Solvent	pH	Vol. in litres	Total units $\times 10^{-3}$
Filtrate	Water	6·0	240	2060
Percolate 1	Water	6·8	70	—
Percolate 2		6·9	70	35
Percolate 3		6·4	75	112
Percolate 4 and water wash		6·2	35	84
Eluate 1	Acetone (60%, v/v, in water)	6·2	35	126
Eluate 2		6·6	2	100
Eluate 3		6·9	16	1410
Eluate 4		7·0	4	112
Total recovered				1979

buffer, pH 6·0 (132 g. KH_2PO_4/l. + 15 ml. 40% (w/v) NaOH/l.), until the pH of the percolate rose to 6·0. Usually 40–50 l. of buffer were required. The following morning percolation with a few litres of the buffer was continued to check that the pH of the percolate had not changed appreciably. The phosphate buffer was then washed out of the column with 50 l. of distilled water, when the pH of the percolate usually rose to 6·4.

Adsorption and elution of cephalosporin N. The clarified culture fluid (240 l.), acidified to pH 5·8–6·0, was percolated down the column at a rate of 2 l./min. The pH of the effluent (which tended to rise) was checked frequently and kept at about 6·0 by adjusting the pH of the culture fluid being added to the column. The percolate was collected in three fractions of about 70 l. and one of 35 l. The column was then washed with 10 l. of distilled water and elution was begun with a 60% (v/v) solution of acetone in water. The eluate was collected in one fraction until a marked increase in the brown colour of the effluent was noted. The main band of cephalosporin N was closely associated with this pigment. The activity of material in eluate 3 (Table 4) was 4 units/mg. The details of the remainder of the elution are shown in Table 4.

Regeneration of the charcoal. After elution of the cephalosporin N, 0·5 N-NaOH was percolated down the column until the effluent became alkaline. Any cephalosporin P which was adsorbed on the charcoal was eluted during this process. The column was then washed with a mixture of 0·2 N-NaOH and acetone (3:2, by vol.) until the effluent, which at first contained much dark brown pigment, was only a pale-brown colour. Free alkali was removed from the column with distilled water (50 l.) and then N-HCl was passed down it until acid broke through. The column was left in contact with acid until the next batch of culture fluid was ready. It was regenerated many times without serious loss of efficiency.

Alumina columns

Preparation of alumina for column 1. Aluminium oxide (British Drug Houses Ltd., 'For chromatographic analysis') which had previously been used for chromatography, was washed with 0·05 N-NaOH until almost colourless, washed with tap water, and then stirred with an excess of 0·05 N-HCl. Finally it was thoroughly washed with distilled water, filtered and dried in a current of air at 30°. The dry powder was heated to 750° in a muffle furnace for 4 hr., cooled, and sieved to provide a fraction of 60–120 mesh. This material was suspended in distilled water and stirred mechanically, while 11 N-HCl was added from time to time until a glass electrode suspended in the water gave a permanent pH reading of 4·0. It was then dried at 30°.

Preparation of alumina for column 2. New aluminium oxide (British Drug Houses Ltd., 'For chromatographic analysis') was suspended in water and stirred. 11 N-HCl was added from time to time until the pH remained stationary at 4·6. The alumina was dried at 30° and sieved to provide a fraction of 60 to 120-mesh.

Chromatography on column 1. A sample (100 ml.) of eluate 3 from the carbon column was extracted with three lots of butyl acetate (100 ml. each) saturated with water; 79 ml. of aqueous solution remained. Eluate 3 therefore contained approximately 21% (v/v) of acetone. Acetone (26 l.) was added to bring the acetone concentration to 70% (v/v) and some material of low activity, which came out of solution, was allowed to settle and was removed by filtration. The pH of the clear filtrate was adjusted to give a reading of 6·0 (glass electrode) and a sample of this solution was percolated down an alumina column (0·5 × 15 cm.). After the passage of 25 ml. of solution, antibacterial activity was detected in the percolate. From these data a column was constructed containing enough alumina to retain cephalosporin N from 1·25 times the volume of the main bulk of the solution. The column (16 cm. diameter), containing 6 kg. of the alumina suspended in 70% (v/v) acetone, was prepared in a glass tube around which cold ethanol (2°) was circulated. The bulk of the clear filtrate (42 l.), cooled to 2°, was percolated through the alumina at about 10 l./hr. Elution was begun with a cold aqueous solution of acetone (20%, v/v) and continued with cold 0·02 N-NaOH. Eluates were adjusted to pH 6·0 immediately they were obtained. Details of the column are given in Table 5.

Chromatography on column 2. Eluates 12–20 (see Table 5) from the first alumina column were combined and 2·25 l. of acetone were added to bring the acetone concentration to 20% (v/v). A small pilot column was used to determine (as for column 1) the amount of alumina required for chromatography of this solution. Table 6 shows the results obtained when 5 l. of solution were chromatographed on 400 g. of alumina. The column (5 cm. diameter) was surrounded by a glass jacket and cooled with ethanol at 2°. The remainder of the eluate selected from column 1 was chromatographed separately.

Table 5. *Chromatography of cephalosporin N on alumina. Column* 1

For details see text.

Fraction	Solvent	pH	Vol. in litres	Units/mg.	Total units ($\times 10^{-3}$)
Charcoal eluate 3	Acetone (70%, v/v)	6·0	42	—	1360
Percolate		5·8	42	—	—
Eluate: 1	Acetone (20%, v/v)	5·6	2	—	—
2		5·7	2	—	36
3		4·8	2	—	38
4		4·3	2	—	22
5		4·2	2	—	11
6		4·0	1	—	3·6
7	0·02 N-NaOH	4·1	1	—	6·7
8		4·1	1	—	17
9		4·1	1	—	27
10		4·2	1	—	33
11		4·3	1	16	37
12		4·2	1	17·7	45
13		4·4	1	24·4	74
14		4·5	1	23·0	84
15		4·5	1	31·0	135
16		4·4	1	33·5	158
17		4·3	1	32·0	148
18		4·5	1	21·5	112
19		4·8	1	18·8	95
20		5·0	1	20	99
21		5·2	1	13·3	67
22		5·4	1	—	39
Total recovered					1286

Table 6. *Chromatography of cephalosporin N on alumina. Column* 2

For details see text.

Fraction	Solvent	pH	Vol. in litres	Units/mg.	Total units ($\times 10^{-3}$)
Eluates 12–20 from previous column	Acetone (20%, v/v)	6·0	5	—	370
Percolate		5·1	5	—	—
Eluate: 1	Water	4·7	0·5	—	—
2		4·6	0·5	—	7·5
3	0·01 N-NaOH	4·6	0·5	25·1	16
4		4·7	0·5	36·0	23
5		4·9	0·5	34·5	25
6		5·2	0·5	31·5	23
7		5·0	0·5	39·5	33
8		5·2	0·5	36·5	32
9		5·1	0·5	25·4	27
10		5·6	0·5	24·5	25
11		5·6	0·5	—	25
12		6·4	0·5	—	22
13		6·9	0·5	—	20
14		7·8	0·5	—	16
Total recovered					298

Approximate determination of the dry weight of eluate fractions. Samples of the eluates were measured into weighed glass tubes and dried in a stream of air while the tubes were maintained at 100°. Owing to decomposition, dry weights estimated by this method were liable to be lower than those obtained by drying *in vacuo* at 20°.

Countercurrent distributions

Solvents. 'AnalaR' phenol and 'AnalaR' CCl_4 were used throughout.

Determination of antibacterial activity. System 1. Samples (0·2 ml.) were taken from even-numbered fractions of the first withdrawn series (aqueous phase) and from the second withdrawn series (phenol phase), and added to 2 ml. of 0·1 M sodium phosphate buffer, pH 7·4. The solutions were extracted twice with CCl_4 (4 ml.) and the final volume of the aqueous phase was noted. Further dilutions to give solutions containing 1–4 units/ml. were made with 0·05 M sodium phosphate buffer, pH 6·8. These solutions were assayed against *Salm. typhi*.

System 2. Water (40 ml.) was added to the combined phases of each fraction (20 ml. each), when a single phase resulted. Samples of these solutions were diluted until they contained 1–4 units/ml. and assayed against *Kleb. pneumoniae*.

Determination of dry weight. System 1. (1) *First withdrawn series (aqueous phase).* CCl_4 (10 ml.) and 0·3 N-$Ba(OH)_2$ (0·175 ml.) were added to samples (5 ml.) taken from every fourth member of the first withdrawn series. The mixture was shaken to establish equilibrium and the pH of the aqueous phase was adjusted to 8·2–8·6 with $Ba(OH)_2$ if necessary. The layers were separated and the aqueous phase was again extracted with CCl_4 (10 ml.). Sufficient 0·1 N-H_2SO_4 (usually 0·12 ml.) was added to the aqueous phase to bring its pH to 6·0–6·4 and a third extraction with CCl_4 (10 ml.) was made. The final volume was measured and the aqueous solution centrifuged to free it from precipitated $BaSO_4$. Samples (3 ml. each) from the clear supernatant were evaporated to dryness *in vacuo* at 30–35° in weighed tubes. Blank determinations on 3 ml. of the aqueous phase gave a dry weight of less than 0·1 mg.

(2) *Second withdrawn series (phenol phase).* From every second fraction of the second withdrawn series a 5 ml. sample was added to a mixture of water (5 ml.) and CCl_4 (20 ml.). 0·3 N-$Ba(OH)_2$ (1–2 ml.) was added until the pH of the aqueous phase at equilibrium was 8·2–8·6. The mixture was centrifuged and the upper (aqueous) layer removed. The lower layer (phenol–CCl_4) was shaken with water (2 ml.) and centrifuged. The upper layer was added to the first aqueous extract and the bottom layer was discarded. The combined aqueous extracts, still at pH 8·2–8·6 were extracted with CCl_4 (10 ml.). 0·1 N-H_2SO_4 was then added to bring the pH to 6·0–6·5 and a final extract made with CCl_4 (10 ml.). The final volume was measured (usually 8·2–8·5 ml.) and the solution centrifuged to remove the precipitated $BaSO_4$. Samples (3 ml. each) were then evaporated to dryness *in vacuo* at 30–35° in weighed tubes.

Ninhydrin determinations. System 1. Samples (0·2 ml. each) of the solutions prepared for estimations of dry weight were added to 0·5 ml. ninhydrin solution (Moore & Stein, 1948). The colour density at 570 mμ. was measured after heating the mixture at 100° for 20 min. (Newton & Abraham, 1950). The same colour densities were obtained from samples of the first withdrawn series whether the solvent was removed or not. No attempt was made to relate the colour density to the dry weight of the samples.

Recovery of cephalosporin N from countercurrent distribution fractions. System 1. The following operations were carried out in a cold room at 2–3°. The selected fractions from the first withdrawn series were pooled, the pH of the solution was brought to 7·0–7·2 by the addition of 0·3 N-$Ba(OH)_2$, one vol. of CCl_4 added, and the mixture shaken. After the phases had separated, the CCl_4 containing most of the phenol originally present in the aqueous phase was drawn off. The pH of the aqueous phase was adjusted to 8·3 and it was extracted twice with one vol. of CCl_4 to remove the 2:4:6-trimethylpyridine. Finally the aqueous phase was acidified to pH 6·5 and extracted once more with one vol. of CCl_4. The solution was then filtered to remove any $BaSO_4$ which remained and any residual CCl_4 was removed *in vacuo*. The solution was then freeze-dried. The product was dissolved in water (1–5 ml.) and acetone was added until its concentration was 95% (v/v). The mixture was centrifuged and the supernatant removed. The sticky precipitate of cephalosporin N was ground under dry acetone, when it formed a fine white powder. It was then centrifuged and dried *in vacuo*.

Distribution in system 1 *at* 3°. (*a*) The starting material was a preparation of cephalosporin N barium salt (38 units/mg.) obtained by distributing material from an alumina column in system 1 at room temperature. This material (approx. 400 mg.) was dissolved in 30 ml. of upper layer and 20 ml. of lower layer of the solvent system. 2:4:6-Trimethylpyridine (0·5 ml.) was added to bring the pH of the upper layer to 6·1 at equilibrium. The mixture was transferred to a glass countercurrent distribution machine (Craig, Hausmann, Ahrens & Harfenist, 1951), and eighty transfers of the upper layer were carried out. Withdrawals of the aqueous phase were made from tube 15 and after sixty-four transfers fresh upper layer was no longer added to tube 0. In this way two withdrawn series remained at the end of the distribution, sixty-five fractions (30 ml.) of the aqueous phase forming the first withdrawn series and sixteen (0–15) fractions (20 ml.) of phenol phase forming the second withdrawn series. Immediately the distribution was finished, ninhydrin determinations were carried out on samples of the first withdrawn series. Fractions 28–58 from this series were pooled after 5 ml. samples had been removed from the even-numbered fractions. They yielded 120 mg. of barium salt assaying at 55 units/mg.

(*b*) The starting material in this experiment was 5·7 g. cephalosporin N sodium salt (28 units/mg.), prepared by chromatography on alumina. To convert it into the barium salt it was dissolved in a mixture of 75 ml. of each layer of a solvent system made up from phenol saturated with water (250 ml.), water saturated with phenol (250 ml.), 2:4:6-trimethylpyridine (20 ml.) and 10 N-H_2SO_4 (14 ml.). 10 N-H_2SO_4 (0·5 ml.) was added to bring the pH of the aqueous layer to 5·6 at equilibrium. The two layers were separated and the aqueous phase was discarded. The phenol phase was shaken with 25 ml. of fresh aqueous phase, which was again discarded after separation of the layers. The phenol phase was shaken with water (25 ml.) and 0·3 N-$Ba(OH)_2$ was added until the pH of the aqueous phase was 8·0. CCl_4 (225 ml.) was added in 75 ml. portions, the equilibrium reaction of the aqueous phase being adjusted back to pH 7·5 with 0·3 N-$Ba(OH)_2$ (in all 190 ml.) after each addition. The lower layer was discarded and the aqueous layer, after its pH had been adjusted to 8·3 with $Ba(OH)_2$, was extracted twice with CCl_4 (250 ml.). The aqueous phase was then brought to pH 6·4 with H_2SO_4 and extracted again with CCl_4 (250 ml.). It was filtered, freed from traces of CCl_4 *in vacuo*, and freeze-dried to yield 5 g. of cephalosporin N barium salt (30 units/mg.).

The barium salt of cephalosporin N (4·4 g.) was added to 60 ml. of upper layer and 40 ml. of lower layer of system 1; 0·025 ml. of 10 N-H_2SO_4 was required to restore the equilibrium reaction of the aqueous phase to pH 6·1. The aqueous phase (30 ml.) and 20 ml. of the phenol phase were put into tubes 0 and 1 of the countercurrent distribution apparatus. Additions of 2:4:6-trimethylpyridine or H_2SO_4 had to be made to the fractions during the first four transfers, but thereafter the pH of the aqueous phases remained constant. Ninety-seven transfers were carried out, withdrawals of the aqueous phase being made from tube 16. Colorimetric ninhydrin determinations on samples of fractions 1–31 and 33–57 of the withdrawn series were made while the distribution was in progress and on fractions 59–77 immediately after the distribution was finished. It was thus possible to decide which fractions to pool for recovery of cephalosporin N, within 30 min. of the end of the distribution. Fractions 20–52 were pooled, freed from solvent and concentrated as described above. They yielded 700 mg. of barium salt assaying at 58 units/mg. The whole experiment was completed in 36 hr.

Evolution of CO_2. This was measured in the apparatus described by Abraham *et al.* (1949). Preparations of cephalosporin N (20–40 mg.) were dissolved in water (1 ml.), enough N-H_2SO_4 was added to lower the pH to 2·0, and the solution was diluted with 0·5 N-H_2SO_4 to 2·5 ml. In control experiments 0·8 mole of CO_2 was obtained from 1 mole of sodium benzylpenicillin under these conditions.

Inactivation with penicillinase. Cephalosporin (40 mg., 32 units/mg.) was dissolved in water (10 ml.) at 25°. A glass electrode was immersed in the solution. A dialysed solution of penicillinase, provided by the Distilers Company (Biochemicals) Ltd., Liverpool (1 ml., containing 2·8 mg. of solid), was added. 0·01 N-NaOH was run into the stirred solution at a rate such that the pH was maintained at 7·0. The rate was constant (1 ml./25 min.) during much of the reaction. After 2·5 hr., when 3·7 ml. 0·01 N-NaOH had been added, there was no further change in pH.

A solution (10 ml.) of sodium benzylpenicillin (10 mg.) absorbed 0·01 N-NaOH under these conditions at an initial rate which was 1·7 times the rate with cephalosporin N.

Iodometric assay. This was done by a method similar to that of Alicino (1946), except that the inactivation by alkali was carried out in 0·4 N-NaOH for 30 min. at 17° and the titration with sodium thiosulphate was carried out 15 min. after the addition of iodine; 3 mg. of sodium benzylpenicillin and 150–300 units of cephalosporin N were used in each titration. For five titrations on a single sample of cephalosporin N (mean = 5·59) the S.D. was 0·108. For four different samples varying in activity from 3·5 to 28 units/mg. the coefficient of correlation between antibacterial activity and differential iodine absorption was 0·83.

Hydroxylamine assay. This was carried out by a method similar to that of Ford (1947), except that ethanol (0·33 vol.) was added to the solution 10 min. after the addition of $FeCl_3$ solution, and the colour density was then read in a Spekker photoelectric absorptiometer, against the corresponding penicillinase-treated control, using a blue-green (Ilford 603, transmitting 470–520 mμ.) filter. For six assays of a single sample (mean = 0·444) the S.D. was 0·007. For four different samples varying in activity from 3·5 to 28 units/mg. the coefficient of correlation between antibacterial activity and the differential Spekker reading was 0·71. Interfering substances appeared to be present in some samples of cephalosporin N, however, since the variation between samples was much greater than the variation in repeated assays of a single sample.

The pigment formed in this procedure from cephalosporin N, unlike that from benzylpenicillin (Baker, Dobson & Martin, 1950) could not be extracted from aqueous solution by *iso*amyl alcohol or butanol.

SUMMARY

1. Cephalosporin N is an acidic antibiotic produced by a strain of *Cephalosporium*. It is inactivated by the enzyme penicillinase but differs strikingly from the common penicillins in its antibacterial activity and hydrophilic character.

2. Cephalosporin N has been purified by chromatography on charcoal and alumina and by countercurrent distribution in a phenol–water system containing 2:4:6-trimethylpyridine as a carrier. The resulting product appears to be at least 75% pure.

3. The acid-base properties and solubility of cephalosporin N, together with its chemical reactivity, suggest that it is a penicillin containing an aminodicarboxylic acid in the side chain.

Our thanks are due to the staff of the Medical Research Council's Antibiotics Research Station, Clevedon, for the supply of fermentation fluid containing cephalosporin N, to Miss N. Smith and her staff who were responsible for the biological assays at Clevedon, and to Miss A. E. Scott, Miss C. M. Hallworth, Mrs A. Savory, Mr D. F. Phillpotts, and Mr D. Gazzard, for technical assistance. We are also indebted to Mrs A. Giles and to Mr O. Boys for technical assistance at Oxford.

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Degradation, Structure and some Derivatives of Cephalosporin N

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Abraham, Newton & Hale (1954) suggested that cephalosporin N was a penicillin containing an aminodicarboxylic acid in the side chain. This paper gives further information about the structure of the molecule.

Elementary analysis of a barium salt of cephalosporin N, which, though amorphous, was probably not very far from pure (Abraham *et al.* 1954), gave the following result: C, 38·2; H, 5·9; N, 8·7; S, 6·9%. The C:S ratio was therefore 14 or 15:1 and the N:S ratio 3:1. Assuming that cephalosporin N contained a normal penicillin nucleus ($C_9H_{11}O_4N_2S$), five or six carbon atoms and one nitrogen atom remained for the side chain. Since the substance showed only end absorption in the ultraviolet region, the side chain appeared to contain no conjugated double bonds.

These conclusions were supported by the products obtained on degradation of the molecule. The course of the degradations was followed by chromatography and electrophoresis on paper, while ion-exchange columns and the four-compartment cell for ionophoresis described by Synge (1951) were used in isolating some of the products. The products were usually isolated from partially purified material, but their formation from cephalosporin N itself was confirmed by work with the purest preparation.

When subjected to ionophoresis on paper in a collidine acetate buffer at pH 7·0, cephalosporin N (detected by antibacterial activity or the ninhydrin reaction) migrated towards the anode at almost the same rate as benzylpenicillin (Fig. 1, spots 1 and 2). On heating at 100° in aqueous solution at pH 2 for 1 hr., carbon dioxide was evolved. This solution contained a penilloic acid, which migrated like the antibiotic on ionophoresis (Fig. 1, spot 3), together with some neutral material. Addition of mercuric chloride to this solution at pH 6 yielded a precipitate of a mercaptide, from which crude penicillamine (I) was obtained by decomposition with hydrogen sulphide. On warming with acetone this gave the crystalline hydrochloride of 2:2:5:5-tetramethylthiazolidine-4-carboxylic acid (II) (m.p. 198°; $[\alpha]_D^{20} + 94°$). Decomposition of (I) by boiling with dilute hydrochloric acid resulted in the formation of D-penicillamine hydrochloride (Abraham, Baker, Chain & Robinson, 1949). Addition of 2:4-dinitrophenylhydrazine in hydrochloric acid to the supernatant from the penicillamine mercaptide produced only a very small precipitate, part of which was glyoxal bis-2:4-dinitrophenylhydrazone. In this, the degradation of cephalosporin N differed from that of other penicillins. It appeared probable that an aldehyde derived from the penilloic acid of cephalosporin N formed a water-soluble 2:4-dinitrophenylhydrazone.

On more vigorous hydrolysis (N hydrochloric acid for 6 hr.) the penilloic acid from cephalosporin N was broken down. Ionophoresis on paper and development with ninhydrin then showed the presence of a new acidic substance, which had migrated about 1·6 times as fast as the penilloic acid towards the anode (Fig. 1, spot 4), together with an increased amount of neutral material. The new acidic substance (Fig. 2, spot 1) travelled further than glutamic acid on paper chromatograms run with butanol–acetic acid (Woiwod, 1949). It was accompanied by glutamic and aspartic acids in hydrolysates of crude cephalosporin N, but not in hydrolysates of the purest preparation. This acid was separated from the neutral material formed on hydrolysis by ionophoresis in the four-compartment cell. It was then purified by chromatography on the weakly basic ion-exchange resin Amberlite IR 4B, eluting with ammonium acetate buffer. After

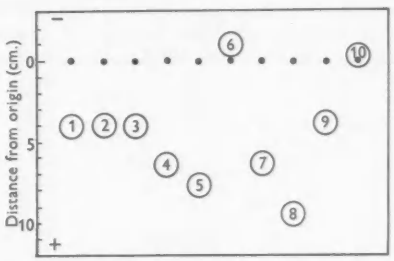

Fig. 1. Positions of derivatives of cephalosporin N after ionophoresis on paper, at pH 7·0 in collidine–acetic acid buffer. Spots developed with ninhydrin. 1, Benzylpenicillin; 2, cephalosporin N; 3, cephalosporin N penilloic acid; 4, α-aminoadipic acid hydrochloride; 5, penicillaminic acid; 6, α-aminoadipylmonoaminoacetaldehyde; 7, α-aminoadipylmonoglycine; 8, cephalosporin N penicilloic acid; 9, cephalosporin N penillic acid; 10, cephalosporin N penillamine.

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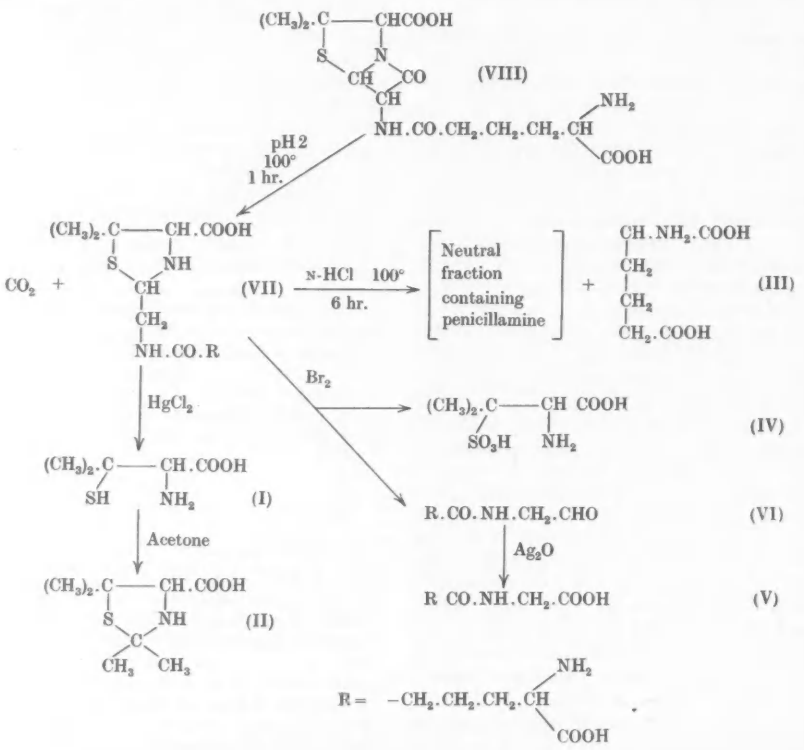
crystallization from water it was found to have the composition $C_6H_{11}O_4N$ (m.p. 207–208°; $[\alpha]_D^{20} - 26°$ in 6N-HCl). Electrometric titration showed the presence of two acidic groups of pK 2·2 and 4·4 respectively and a basic group of pK 9·8. The properties of the substance indicated that it was D-α-aminoadipic acid (III). An X-ray powder photograph of the substance was reported by Dr D. Hodgkin to be identical with a photograph of synthetic D-α-aminoadipic acid supplied by Dr J. P. Greenstein. The synthetic compound ($[\alpha]_D^{25} - 25°$ in 5NHCl) is assumed to have the D configuration because its chloroacetyl derivative was prepared by subjecting chloroacetyl-DL-α-aminoadipic acid to the asymmetric action of acylase I, and because it was not oxidized by the L-amino acid oxidase from *Crotalus adamanteus* (Greenstein, Birnbaum & Otey, 1953). Its rotation in 5N-HCl has the same sign as that of D-glutamic acid and is of similar magnitude.

The neutral fraction from the four-compartment cell yielded an acidic amino acid on oxidation with bromine water (Fig. 1, spot 5). This was separated from residual neutral material by further ionophoresis in the four-compartment cell and on crystallization from aqueous acetone yielded prisms of penicillaminic acid (β-sulphovaline) (IV).

The penilloic acid formed by hydrolysis of cephalosporin N in hot aqueous solution at pH 2 had an isoelectric point in the neighbourhood of pH 3·5. When kept at this pH it remained in the specimen compartment of the four-compartment cell, while impurities of stronger acidity migrated into the acetic acid compartment. Oxidation of the penilloic acid with bromine water resulted in the formation of penicillaminic acid and a neutral aldehyde (Fig. 1, spot 6) which were separated by ionophoresis. Oxidation of the aldehyde with freshly precipitated silver oxide yielded an acid (Fig. 1, spot 7) which migrated into the acetic acid compartment of the four-compartment cell. This compound crystallized from aqueous ethanol in small white prisms, m.p. 213–214°. Its composition and equivalent weight were consistent with the formula $C_8H_{14}O_5N_2$. It contained a basic group with pK 9·9 and two acidic groups with pK values of 3·8 and < 3·0 respectively. On paper chromatograms run with butanol–acetic acid and developed with



ninhydrin (Fig. 2, spot 3) it appeared in a position close to that of glycine. On hydrolysis with 6N hydrochloric acid at 105° it yielded about equimolecular amounts of a neutral substance and an acidic substance which behaved like glycine and α-aminoadipic acid respectively on paper chromatograms. After treatment with 1:2:4-fluorodinitrobenzene (FDNB) (Sanger & Thompson, 1953), hydrolysis resulted in the formation of glycine and of a substance which behaved like dinitrophenyl-α-aminoadipic acid (DNP-α-aminoadipic acid) when chromatographed on paper in the system of Blackburn & Lowther (1951). The original acid was therefore an α-aminoadipylmonoglycine, and the aldehyde from which it was derived was an α-aminoadipylmonoaminoacetaldehyde.

The dissociation constants of the α-aminoadipylmonoglycine, compared with those of α-aminoadipic acid and other model compounds, indicated that the δ-carboxyl group of α-aminoadipic acid was involved in a peptide bond. The pK of the basic group of an α-amino acid is reduced by about 1·5 units when the α-carboxyl group is involved in a peptide linkage, because the effect of the electronegative CO group is no longer opposed by the charge on the carboxylate ion (Neuberger, 1937). The influence of the CO group, however, diminishes rapidly when it is removed from the α-position. Thus the pK values of the amino groups of glutamic acid, γ-L-glutamyl-L-alanine, and α-L-glutamyl-L-valine were found to be 9·7, 9·6, and 8·1 respectively. Since the pK of the amino group of the α-aminoadipylglycine was very close to that of α-aminoadipic acid, the former appeared to be D-δ-amino-δ-carboxyvalerylglycine (V) and the aldehyde from which it was derived to be D-δ-amino-δ-carboxyvalerylaminoacetaldehyde (VI).

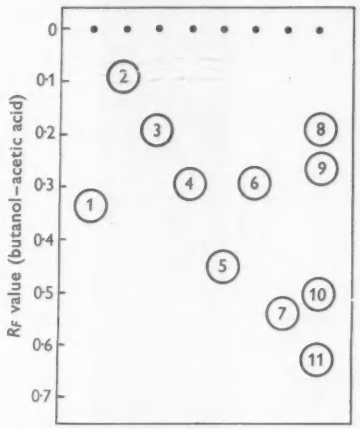


Fig. 2. Single dimension paper chromatogram of derivatives of cephalosporin N and reference amino acids in butanol–acetic acid. Spots developed with ninhydrin. 1, α-Aminoadipic acid hydrochloride; 2, penicillaminic acid; 3, α-aminoadipylmonoglycine; 4, cephalosporin N penillamine; 5, cephalosporin N *S*-benzylpenillamine; 6, cephalosporin N *S*-benzylpenillamine sulphone; 7, *S*-benzylpenicillamine sulphone; 8, glycine; 9, glutamic acid; 10, valine; 11, leucine.

These results indicate that the penilloic acid has the structure (VII) and that cephalosporin N is therefore (D-4-amino-4-carboxy-*n*-butyl)penicillin (VIII). This structure is in accordance with the fact that the purest preparations of cephalosporin N react with FDNB to form a DNP-derivative which yields DNP-α-aminoadipic acid on hydrolysis, and that the pK of the basic group of cephalosporin N (Fig. 3) is very close to that of α-aminoadipic acid itself.

In alkaline solution above pH 11, cephalosporin N was rapidly inactivated, and electrometric titration revealed that a new ionizable group had appeared with pK 4·7. Ionophoresis on paper at pH 7·0 showed that the product migrated faster towards the anode than α-aminoadipic acid (Fig. 1, spot 8). The new compound was presumably the penicilloic acid (IX), containing three acidic groups, one strong basic group and one weak basic group that was un-ionized at pH 7·0.

When an aqueous solution of cephalosporin N is kept at pH 2·7 and at 37° for 2 hr. there is a loss of antibacterial activity, an increase in dextrorotation, and a change in the ultraviolet absorption spectrum which results in the appearance of strong absorption at 240 mμ. (Fig. 4). Electrometric titration shows that a new basic group has been formed with a pK of about 7·6. These changes are typical of those occurring in a penicillin → penillic acid transformation.

The penillic acid from cephalosporin N (Fig. 1, spot 9) was purified by chromatography on a buffered column of Amberlite IR4B. It was obtained as an amorphous white powder ($[\alpha]_D^{20} + 354°$

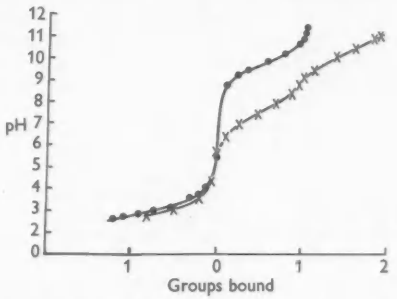


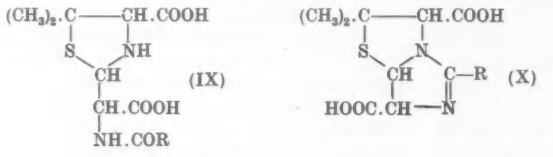
Fig. 3. Electrometric titration curves at 20°. ●—●, Cephalosporin N barium salt (58 units/mg.); ×—×, cephalosporin N penillic acid.

in water; $\lambda_{max.}$ 240 mμ., $E^{1\%}_{1\,cm.}$ 140). It contained three acidic groups, the weakest having a pK of 3·1, and two basic groups with pK values of 7·6 and 10·3 respectively (Fig. 3). The results of elementary analysis were in approximate agreement with those expected of a hydrate of $C_{14}H_{21}O_6N_3S$ (X).

The penillic acid yielded the same products as cephalosporin N on hydrolysis with N hydrochloric acid at 100°. A paper chromatogram of a hydrolysate which had been treated with bromine water showed the presence of penicillaminic acid and α-aminoadipic acid in approximately equimolecular proportion, together with a trace of glycine (Fig. 5). A hydrolysate of sodium benzylpenicillin to which one molecular proportion of α-aminoadipic acid had been added behaved in a very similar manner (Fig. 5).

Addition of mercuric chloride to a solution of the penillic acid resulted in the immediate precipitation of a white mercaptide. Decomposition of the latter with hydrogen sulphide yielded an amorphous neutral substance (Fig. 1, spot 10), which was laevorotatory, gave a strong positive test for a free thiol group, and showed a spot on paper chromatograms run with butanol–acetic acid which had an R_F value slightly less than that of α-aminoadipic acid (Fig. 2, spot 4). This compound had the properties of a penillamine (XI). It could be precipitated from aqueous solution as a picrate, which facilitated its preparation from crude penillic acid.

Benzylation of the penillamine yielded an *S*-benzylpenillamine which crystallized from warm



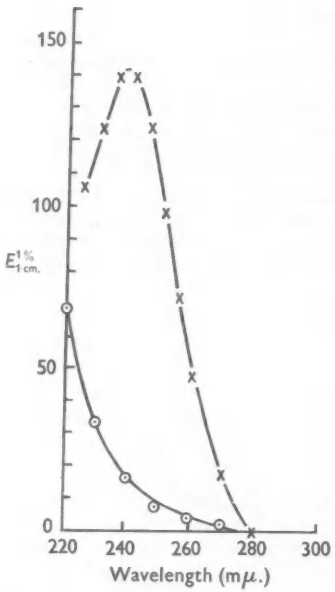


Fig. 4. Ultraviolet absorption spectra in water. ⊙—⊙, Cephalosporin N barium salt (58 units/mg.); ×—×, cephalosporin N penillic acid.

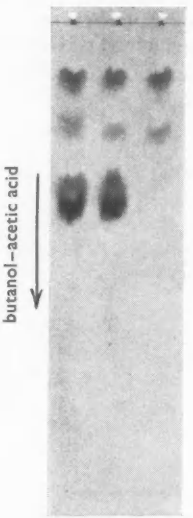


Fig. 5. Chromatogram of products formed from cephalosporin N penillic acid and benzylpenicillin on hydrolysis with N-HCl and oxidation with bromine water. 1, Cephalosporin N penillic acid; 2, benzylpenicillin plus α-aminoadipic acid; 3, benzylpenicillin. The top spot is penicillaminic acid and the bottom spot α-aminoadipic acid.

water in fine needles, m.p. 196–198°. The equivalent weight and elementary composition of this substance agreed with the formula $C_{20}H_{27}O_4N_3S$, $\frac{1}{2}H_2O$. Its ultraviolet absorption spectrum showed only end absorption and a plateau in the region 250–265 mμ. ($E^{1\%}_{1cm.}$ 9·0) due to the presence of a benzyl group. Titration showed the presence of two acidic groups with pK values of 2·5 and 2·2, and two basic groups with pK values of 7·0 and 10·0 respectively (Fig. 6). On paper chromatograms run with butanol–acetic acid its R_F value was slightly less than that of valine (Fig. 2, spot 5). In the ninhydrin–carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) for the determination of amino acids, it yielded one molecular proportion of carbon dioxide.

The *S*-benzylpenillamine remained largely unchanged on treatment with hot dilute hydrochloric acid for several hours. On oxidation with hydrogen peroxide in formic acid (Sanger & Thompson, 1953) it formed a compound (presumably a sulphone) with an R_F value slightly greater than that of glutamic acid (Fig. 2, spot 6). On treatment with bromine water, both the *S*-benzylpenillamine and its sulphone were broken down. Paper chromatograms of the products showed the presence of one spot in the position of benzylpenicillamine sulphone (Fig. 2, spot 7) and one spot in the position of α-aminoadipic acid.

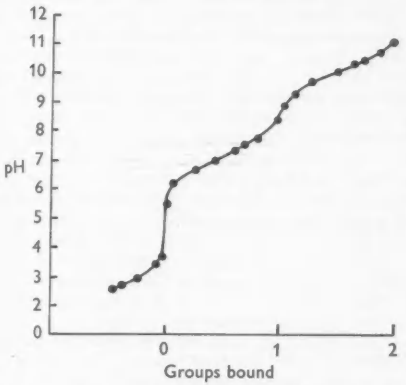


Fig. 6. Electrometric titration curve of cephalosporin N *S*-benzylpenillamine at 20°.

Crude preparations of cephalosporin N yielded aspartic and glutamic acids as well as α-aminoadipic acid on hydrolysis. It therefore seemed possible that aspartyl and glutamyl derivatives of the penicillin nucleus were formed by the *Cephalosporium* (Newton & Abraham, 1953). Further work, however, has produced no evidence of the existence of more than one active substance in crude material, and it is perhaps significant that the glutamic acid from this material, unlike the aminoadipic acid, has the L configuration. D-α-Aminoadipic acid does not appear to have been isolated previously from a natural source although it has been prepared synthetically (Greenstein *et al.* 1953), but L-α-aminoadipic acid is involved in the metabolism of lysine in the guinea pig (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1948) and in *Neurospora* (Mitchell & Houlahan, 1948). In crude cephalosporin N, α-aminoadipic acid is present in compounds other than the antibiotic itself. These compounds are being investigated further.

Cephalosporin N reacts readily with certain acid halides, acetic anhydride, and phenyl isocyanate, in sodium bicarbonate solution, to form derivatives which do not contain a free amino group. These derivatives still contain the penicillin nucleus because they are rapidly inactivated by penicillinase, but they differ greatly from the parent compound in solubility and antibacterial activity. They can be extracted from an aqueous solution at pH 2·5 into ethyl acetate, or butanol, and re-extracted into water at pH 6·0. They have a higher activity than cephalosporin N against *Staphylococcus aureus* and a lower activity against *Salmonella typhi*. None of the derivatives has yet been purified, but Table 1 shows the change in antibacterial activity of an aqueous solution of crude cephalosporin N (28 units/mg.) when they are formed, and the relative activities of the products after passage through an organic solvent.

These derivatives of cephalosporin N resemble benzylpenicillin more closely than cephalosporin N itself in some of their physical and antibacterial

Table 1. *Changes of antibacterial activity in the formation of derivatives of cephalosporin N*

Derivative formed from	Product	Final/initial activity of solution against		(Activity against *Staph. aureus*/ Activity against *Salm. typhi*) of extracted product
		Staph. aureus	*Salmonella typhi*	
—	Benzylpenicillin	—	—	140
—	Cephalosporin N	—	—	1
FDNB	DNP-cephalosporin N	5	—	1000
Benzoyl chloride	Benzoylcephalosporin N	2·9	0·10	31
Benzyloxycarbonyl chloride	Benzyloxycarbonylcephalosporin N	2·1	0·35	7
Phenyl isocyanate	Phenylcarbamylcephalosporin N	2·2	0·23	11
Acetic anhydride	Acetylcephalosporin N	1	0·14	7

properties. Their absolute activity against either *Staph. aureus* or *Salm. typhi*, however, appears to be less than that of benzylpenicillin. This fact may be associated with the presence of a carboxyl group in the side chain. Coghill, Stodola & Wachtel (1949) found that azo derivatives of *p*-hydroxybenzylpenicillin in which the substituent carried an acidic group were less active against *Staph. aureus* than derivatives in which the substituent was non-polar.

EXPERIMENTAL

Ionophoresis on paper. This was carried out in an apparatus similar to that described by Flynn & Mayo (1951) using sheets of Whatman no. 1 paper 36 cm. long. The buffer (pH 7·0) was prepared by adding 36·3 ml. of commercial collidine (distilled under N_2; b.p. 54°/12 mm.) and 185 ml. of N acetic acid to 3·86 l. of water. The apparatus was run at 500 V for 3 hr. Substances with no net charge at pH 7·0 were carried about 1 cm. towards the cathode by endosmosis.

Four-compartment cell. This was similar to that described by Synge (1951) and was fitted with a cooling coil carrying cold tap water. In most cases the potential was raised to 500 V and the current fell to about 35 mA towards the end of the experiment.

Electrometric titrations. These were carried out in the manner described by Newton & Abraham (1952).

Antibacterial activity. This was measured by a diffusion method on plates seeded with the test organism (Abraham *et al.* 1954).

Isolation of penicillamine. Cephalosporin N (500 mg.; 42 units/mg.) was dissolved in water (8 ml.) and 2 N-HCl (0·63 ml.) was added to bring the pH of the solution to 2·0. The solution was refluxed in a stream of N_2 for one hr. Passage of the N_2 through 0·3 N-$Ba(OH)_2$ resulted in the precipitation of $BaCO_3$. Titration of the remaining $Ba(OH)_2$, using phenolphthalein as an indicator, showed that CO_2 corresponding to 8·8 ml. of 0·1 N-HCl had been evolved. The pH of the hydrolysate was adjusted to 6·0 and a saturated aqueous solution of $HgCl_2$ (25 ml.) was added. Within a few minutes a flocculent precipitate formed which was separated by centrifuging. After washing with 5 ml. of a solution of $HgCl_2$ (70% saturated) the precipitate was suspended in water (5 ml.) and decomposed with H_2S. The HgS was removed at the centrifuge and the supernatant solution evaporated *in vacuo*. The residue (68 mg.) gave a strong positive reaction for a free thiol group with sodium nitroprusside. It was heated under reflux with acetone (10 ml.) for 30 min., and the hot solution filtered and evaporated to dryness. A crystalline residue remained which consisted of 2:2:5:5-tetramethylthiazolidine-4-carboxylic acid (33 mg.). This was redissolved in hot acetone, the solution concentrated, and 0·05 ml. of 11 N-HCl added. The hydrochloride of 2:2:5:5-tetramethylthiazolidine-4-carboxylic acid (20 mg.) separated from the solution in prisms, m.p. 198°; $[\alpha]_D^{20} + 94°$ in water (*c*, 1·0). (Found: C, 40·6; H, 7·7; N, 6·0; S, 12·4; Cl, 14·8. $C_8H_{15}O_2NS$, HCl, $\frac{1}{2}H_2O$ requires C, 40·9; H, 7·3; N, 6·0; S, 13·6; Cl, 15·1%.) An X-ray powder photograph was identical with a photograph of an authentic sample of the thiazolidine.

The thiazolidine (5 mg.) was dissolved in N-HCl (1 ml.) and the solution boiled for 2 min. Evaporation *in vacuo* yielded a residue which crystallized in long needles and gave a strong positive reaction for a free thiol group with sodium nitroprusside. An X-ray powder photograph of the compound was identical with a photograph of authentic penicillamine.

Isolation of D-α-*aminoadipic acid.* Cephalosporin N (500 mg.; 28 units/mg.) was hydrolysed in N-HCl for 7 hr. at 110° under N_2. The hydrolysate was evaporated to dryness *in vacuo* and the residue separated into acidic and neutral fractions in the four-compartment cell. The acidic fraction (210 mg.) was found by paper chromatography to be a mixture of α-aminoadipic, glutamic and aspartic acids. This material, together with about 50 mg. of similar material from another experiment, was chromatographed on a column (4 × 27 cm.) of Amberlite IR 4B (200–320 mesh). The amino acids were eluted with 0·2 M ammonium acetate buffer, pH 5·0 (Hirs, Moore & Stein, 1952), the eluate being collected in 20 ml. fractions. The rate of flow was adjusted to 20 ml./25 min. The fractions containing the amino acids were located roughly by spotting samples of each fraction (3 × 5·3 μl.) on a sheet of filter paper, heating to 110° for 10 min. to remove ammonium acetate, spraying with ninhydrin solution, and then heating at 105° for 5 min. Blue spots developed from fractions 50–59 and 61–70. Much weaker spots were obtained from fractions 82–86.

Samples (0·05 ml.) from every other fraction over the region where the amino acids had been located, were placed into test tubes (3 × 0·5 in.) and evaporated to dryness *in vacuo*. The ammonium acetate was sublimed by placing the tubes in holes drilled into a blackened aluminium block, which was placed in a desiccator containing dishes of P_2O_5 and KOH. The desiccator was continuously evacuated with a high-vacuum pump for 8 hr. while the block was heated to 40–50° by means of an external infrared lamp (we are indebted to Dr D. Kay for this suggestion). Quantitative ninhydrin determinations on the resulting samples (Moore & Stein, 1948), showed that the mixture had been resolved into three components which accounted for 50% (fractions 50–59), 37·5% (fractions 60–74) and 12·5% (fractions 76–92) respectively of the total. The fractions of the major band, which contained α-aminoadipic acid, were pooled and freed from ammonium acetate buffer (Hirs *et al.* 1952). The residue was dissolved in 4 ml. of hot water and glacial acetic acid was added until the pH of the solution was 4·0. It was then left to crystallize overnight. The crystals were filtered and washed with cold water. The first crop yielded 35 mg. A further 25 mg. were obtained by concentrating the mother liquor and acidifying to pH 3·2. The substance had m.p. 207–208°; $[\alpha]_D^{20} - 26°$ in 6 N-HCl (*c*, 1·0). (Found: C, 44·2; H, 7·0; N, 9·0. $C_6H_{11}O_4N$ requires C, 44·8; H, 6·8; N, 8·7%.) The mixed m.p. with an authentic sample of DL-α-aminoadipic acid (m.p. 198°), kindly provided by Dr E. Work, was 188–190°. The mixed m.p. with an authentic sample of D-α-aminoadipic acid, kindly provided by Dr J. P. Greenstein, was 207–208°.

On descending chromatograms run with butanol–acetic acid, free α-aminoadipic acid was liable to give two separate spots close together. Only the lower spot, however, was obtained when the amino acid was applied to the paper as the hydrochloride, and only the higher spot when it was applied as the ammonium salt.

Isolation of L-*glutamic acid.* The material recovered from fractions 60–74 of the preceding experiment was dissolved in 2 N acetic acid (1·5 ml.) and ethanol (3 ml.) was added to the solution. The L-glutamic acid which separated (33 mg.) had $[\alpha]_D^{20} + 27·9°$ in 6 N-HCl (*c*, 0·8). Crystallization from 11 N-

HCl yielded L-glutamic acid hydrochloride. (Found: C, 33·2; H, 5·2; N, 8·0; Cl, 19·0. $C_5H_{10}O_4NCl$ requires C, 32·7; H, 5·4; N, 7·7; Cl, 19·3%.)

Oxidation of D-α-*aminoadipic acid with chloramine-T.* The procedure was similar to that used by Dakin (1917) for the oxidation of glutamic acid. D-α-Aminoadipic acid (16 mg.) from cephalosporin N was dissolved in N-NaOH (1 ml.) and chloramine-T (29 mg.) was added. The mixture was kept for 5 min. at 20° and then for 15 min. at 50°. Addition of 2:4-dinitrophenylhydrazine in 2N-HCl precipitated an acidic 2:4-dinitrophenylhydrazone (10 mg.) which was crystallized twice from aqueous ethanol. This substance had m.p. 130–131°. The mixed m.p. with a substance obtained in a similar manner from authentic DL-α-aminoadipic acid was 130–131°. The substance appeared to be the 2:4-dinitrophenylhydrazone of glutaric acid semialdehyde. (Found: C, 44·8; H, 4·2; N, 18·4. $C_{11}H_{12}O_6N_4$ requires C, 44·5; H, 4·1; N, 18·9%.)

Isolation of penicillaminic acid. The neutral fraction obtained from the four-compartment cell in the isolation of α-aminoadipic acid was oxidized by passing Br_2 vapour through the solution until Br_2 was present in slight excess. The solution was freeze-dried and the residue dissolved in water (20 ml.). The resulting solution, after adjusting its pH to 3·5 with 2N-NH_3, was placed in the specimen compartment of the four-compartment cell. Current was then passed through the cell for 3 hr., the potential being gradually raised to 500 v, and the pH of the specimen compartment being maintained at 3·5. The contents of the acetic acid compartment were evaporated to dryness and yielded a crystalline residue of penicillaminic acid (65 mg.). This was dissolved in water, decolorized by treatment with charcoal, and recrystallized by the addition of acetone to the solution. Penicillaminic acid separated in the form of white prisms. (Found: C, 30·6; H, 6·0; N, 6·9. $C_5H_{11}O_5NS$ requires C, 30·7; H, 5·6; N, 7·1%.) An X-ray powder photograph was identical with a photograph of authentic penicillaminic acid.

Isolation of δ-amino-δ-carboxyvalerylglycine. Crude penillic acid was prepared from cephalosporin N (42 units/mg.) in the manner described in the following experiment. It was separated from cations by ionophoresis in the four-compartment cell. The penillic acid (470 mg.) was dissolved in water (25 ml.) and the solution heated at 100° in a stream of N_2 for 2 hr. CO_2 was evolved and penilloic acid formed. The pH of the solution was adjusted to 3·0 with 2N-NH_3 and the solution was placed in the specimen compartment of the four-compartment cell. The current was passed until it fell to 50 mA at 250 v. At pH 3·5 most of the penilloic acid remained in the specimen compartment, while impurities of stronger acidity migrated into the acetic acid compartment.

The contents of the specimen compartment (150 mg.) were oxidized by passing a slight excess of Br_2 vapour into the solution. The solution was then stirred with freshly precipitated Ag_2CO_3 until the pH rose to 3·9. After removing the AgBr and excess of Ag_2CO_3 at the centrifuge, N-HCl was added until no further precipitate of AgCl occurred. The solution was centrifuged, and the supernatant, after adjusting its pH to 4·5 with 2N-NH_3, was replaced in the specimen compartment of the cell. Current was passed for 4 hr. until it finally fell to 25 mA at 500 v, the pH being maintained at 4·5. Penicillaminic acid had then migrated into the acetic acid compartment and a neutral aldehyde remained in the specimen compartment.

The solution from the specimen compartment was adjusted to pH 7·0 and stirred at 80° for 2 hr. with Ag_2O freshly precipitated from 400 mg. of $AgNO_3$. Reduction of Ag_2O began almost immediately. The solution was centrifuged and the supernatant was adjusted to pH 3·0 with N-HCl. After removing AgCl at the centrifuge the supernatant was adjusted to pH 7·0 and again placed in the four-compartment cell. Current was passed for 2·5 hr., the potential being raised gradually to 500 v and the pH of the specimen compartment maintained at 7. The contents of the acetic acid compartment were evaporated *in vacuo*, leaving a crystalline residue (20 mg.). This was dissolved in water containing a few drops of glacial acetic acid, and ethanol added until crystallization began. The product separated in clusters of small prisms, m.p. 213–214°. (Found: C, 44·0; H, 6·4; equiv. 230. $C_8H_{14}O_5N_2$ requires C, 44·0; H, 6·4%; equiv. 218.)

The α-aminoadipylmonoglycine could be chromatographed on Amberlite IR 4B (200–320 mesh) at pH 3·5. It was eluted from a column (20 × 1·2 cm.) with 0·8N acetic acid containing enough NH_3 to bring the pH to 3·5. Fractions of 2 ml. were collected and the substance emerged as a single band in fractions 42–48.

Preparation of penillic acid. Cephalosporin N (1 g., 51 units/mg.) was dissolved in water (80 ml.) and the solution adjusted to pH 3·0 with N-HCl (1·4 ml.). The solution was kept at 37° and the rise in the optical density at 240 mμ. was determined at 30 min. intervals. After 3 hr. the value of $E^{1\%}_{1\,cm.}$ had increased from 37 to a maximum of 87. The solution was immediately freeze-dried.

A portion of the resulting crude penillic acid (870 mg.) was chromatographed on a column (34 × 4 cm.) of Amberlite IR 4B (200–320 mesh). Elution was carried out with 0·2M ammonium formate (pH 3·2) containing 0·5% (v/v) thiodiglycol (Hirs *et al.* 1952). Fractions of 30 ml. were collected at 30 min. intervals. The optical density at 240 mμ. and the ninhydrin colour density after removing ammonium formate by sublimation, were determined on samples taken from selected fractions. The penillic acid was located in fractions 30–45. Fractions 36–40 were freeze-dried and the residue dissolved in water and placed in the specimen compartment of the four-compartment cell. The penillic acid, which migrated into the acetic acid compartment of the cell, was recovered by freeze-drying. The product was dissolved in water (5 ml.) and precipitated by the addition of dry acetone (95 ml.). The sticky precipitate was separated by centrifuging and dried to a fine white powder (110 mg.) by grinding under dry acetone. It had $[\alpha]_D^{20} + 354°$ in water (*c*, 0·25). (Found: C, 45·3; H, 6·4; N, 10·2; S, 8·2. $C_{14}H_{20}N_3SO_6$, H_2O requires C, 44·6; H, 5·9; N, 11·2; S, 8·5%.)

In the photometric ninhydrin determination (Moore & Stein, 1948) 100 μg. of the penillic acid gave the same colour density as 29 μg. of leucine.

Preparation of an S-*benzylpenillamine.* Crude penillic acid (400 mg.) from cephalosporin N (40 units/mg.) was dissolved in 20 ml. water and a saturated aqueous solution of $HgCl_2$ (30 ml.) was added. A white precipitate formed and CO_2 was evolved. The precipitate was centrifuged, suspended in water (15 ml.) and decomposed with H_2S. After removing the HgS the supernatant was evaporated *in vacuo*. The amorphous residue (200 mg.) gave a strong positive test for a free thiol group with sodium nitroprusside. The material was dissolved in water (2·5 ml.) and a saturated aqueous solution of picric acid was added until no more precipitate was formed. The oily penillamine picrate was separated from the solution and converted into

the hydrochloride by solution in 0·5 N-HCl (10 ml.) and extraction of the picric acid with ether. Evaporation of the colourless aqueous solution *in vacuo* yielded amorphous penillamine hydrochloride (75 mg.), $[\alpha]_D^{20} - 23°$ in water (*c*, 1·5).

The penillamine hydrochloride was dissolved in liquid NH_3 (15 ml.) and sodium added in small pieces until the solution developed a permanent blue colour. Benzyl chloride (0·026 ml.) was then added and the NH_3 evaporated. The residue was dissolved in water and the pH of the solution adjusted to 5·5 with 2 N-HCl. The solution was extracted with ether to remove excess of benzyl chloride and placed in the specimen compartment of the four-compartment cell. Current was passed for 2·5 hr., until it fell to 30 mA. at 500 V, and the pH of the solution was maintained at 5 to 6. Evaporation of the contents of the specimen compartment *in vacuo* gave a white crystalline residue (55 mg.). Recrystallized from warm water (1 ml.) it formed fine needles, m.p. 196–198°. (Found: C, 58·1; H, 6·7; N, 9·6; S, 7·9; equiv. 410. $C_{20}H_{27}O_4N_3S$, $\frac{1}{2}H_2O$ requires C, 58·1; H, 6·8; N, 10·1; S, 7·7%; equiv. 414.)

In the ninhydrin–carbon dioxide reaction of Van Slyke *et al.* (1941), the CO_2 obtained from 10 mg. of the *S*-benzylpenillamine neutralized 0·46 ml. of 0·1 N-$Ba(OH)_2$. The theoretical amount of CO_2 from one α-aminoadipic acid residue would have neutralized 0·49 ml. of 0·1 N-$Ba(OH)_2$. Under the same conditions no CO_2 was obtained from benzylpenillamine, prepared from benzylpenicillin.

Reaction of cephalosporin N with 1:2:4-fluorodinitrobenzene (FDNB). FDNB (1·5 ml.) was added to a mixture of 4% (w/v) $NaHCO_3$ (24 ml.) and acetone (35 ml.). A sample (2 ml.) of the solution was removed to serve as a blank for determinations of optical density. Cephalosporin N (1 g.; 24 units/mg.) was added to the remainder of the solution at 20°. Samples were removed at intervals and the increase in optical density at 350 mμ., relative to the blank solution, was determined. The reaction was completed in about 45 min. The pH of the solution, which was 8·5 (glass electrode) during the reaction, was adjusted to 6·5 with 2 N-HCl and the acetone was then removed *in vacuo*. When assayed against *Staph. aureus* this solution was found to contain 5 times the total activity of the starting material.

The subsequent operations were carried out at 3°. The solution was acidified to pH 2·8 and extracted 3 times with one vol. of ethyl acetate. The extract was shaken twice with one vol. of water, adjusting the pH to 5·5 with N-NaOH. The resulting aqueous solution was brought to pH 7·0 and freeze-dried. The preparation of DNP-cephalosporin N sodium salt (500 mg.) showed an activity of 200 units/mg. when assayed against *Staph. aureus*, and 0·2 units/mg. when assayed against *Salm. typhi*.

Reaction with benzoyl chloride. Crude cephalosporin N (100 mg., 28 units/mg.) was dissolved in 3% (w/v) aqueous $NaHCO_3$ (6 ml.) and acetone (6 ml.) was added. The solution was cooled to 0°, stirred, and 0·05 ml. benzoyl chloride in acetone (11 ml.) was slowly added. After 20 min. the acetone was removed *in vacuo* and the aqueous solution (adjusted to pH 7·0) extracted with one vol. of ether. The resulting solution (5·5 ml.) contained 1485 units/ml. when assayed against *Staph. aureus*. It was cooled to 0°, its pH adjusted to 2·5 with 2 N-HCl, and extracted twice with one vol. of *n*-butanol. The combined butanol extracts were shaken twice with water (3 ml.), adjusting the pH of the aqueous phase to 6·5. The aqueous solution was extracted with ether and then freeze-dried. The product (65 mg.) showed an activity of 123 units/mg. when assayed against *Staph. aureus* and 4 units/mg. when assayed against *Salm. typhi*.

Reaction with benzyloxycarbonyl chloride. This reaction was carried out in a similar manner to the previous one. Crude cephalosporin N (100 mg.; 28 units/mg.) yielded a solution (5·5 ml.) which showed an activity of 1060 units/ml. against *Staph. aureus*. The product obtained from the butanol extract (71 mg.) showed an activity of 81 units/mg. against *Staph. aureus* and 11 units/mg. against *Salm. typhi*.

Reaction with phenyl isocyanate. This was carried out in a similar manner to the reaction with benzoyl chloride, using 100 mg. of crude cephalosporin N (28 units/mg.). After the reaction, the solution contained 6160 units when assayed against *Staph. aureus* and 645 units when assayed against *Salm. typhi*. The extract from butanol contained 4060 units against *Staph. aureus* and 370 units against *Salm. typhi*.

Reaction with acetic anhydride. Crude cephalosporin N (35 mg., 28 units/mg.) was allowed to react with acetic anhydride (0·01 ml.) under conditions similar to those used in the reactions with acid chlorides. The resulting solution contained 980 units when assayed against *Staph. aureus* and 135 units when assayed against *Salm. typhi*. The extract from butanol contained 800 units against *Staph. aureus* and 120 units against *Salm. typhi*.

SUMMARY

1. Cephalosporin N is hydrolysed by hot dilute acid to a penilloic acid, from which penicillamine may be obtained by treatment with mercuric chloride. More vigorous hydrolysis results in the liberation of D-α-aminoadipic acid.

2. Oxidation of the penilloic acid with bromine water yields penicillaminic acid and a neutral aldehyde. The latter has been oxidized with silver oxide to an α-aminoadipylmonoglycine. The p*K* of the amino group of this compound indicates that α-aminoadipic acid is linked to glycine through its δ-carboxyl group.

3. It is concluded from these results that cephalosporin N is probably (D-4-amino-4-carboxy-*n*-butyl)penicillin.

4. Cephalosporin N is transformed to a penillic acid in aqueous solution at pH 2·7. The penillic acid reacts with mercuric chloride to form a penillamine which has been isolated as a crystalline *S*-benzyl derivative.

5. Cephalosporin N reacts with certain acid chlorides, acetic anhydride, and phenyl *iso*cyanate, to form derivatives which contain no free amino group. These derivatives are more active against *Staphylococcus aureus* and less active against *Salmonella typhi* than cephalosporin N itself.

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Site of Inhibitory Action of Cocaine on Cellular Respiration

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It has long been recognized that cocaine depresses cell metabolism. Niwa (1919) demonstrated that cocaine, in concentration sufficient to anaesthetize the sciatic nerve of a frog, reduced the production of carbon dioxide by the isolated nerve; Felloni (1935) observed that cocaine depressed the oxygen uptake of blood, Wortis (1935) that of brain and, more recently, Cook & McDevitt (1945) have shown that cocaine and other local anaesthetics retarded the respiration of yeast. Cocaine inhibits certain esterases (Broekmeyer, 1924), some dehydrogenases (Bach & Lambert, 1938) and the esterification of phenols, including adrenaline (Torda, 1943*a*, *b*).

Investigations preliminary to a detailed study into the mode of action of cocaine in depressing cellular metabolism have revealed that cocaine inhibits both fermentation and respiration by retarding the metabolism of pyruvate. The results of experiments with *Saccharomyces cerevisiae* showed that depression of alcoholic fermentation could be attributed to inhibition of carboxylase and that growth in the presence of cocaine induced in the yeast a modified response to addition of vitamin B_1 (Ryman & Walsh, 1951, 1952).

Experiments analogous to those with *S. cerevisiae* on fermentation have now been carried out on respiration using *Torulopsis utilis*. Investigations into the effects of cocaine on isolated steps of carbohydrate oxidation have been carried out mainly with enzyme systems prepared from mammalian tissues.

The results here presented show that the toxicity of cocaine towards tissue respiration lies, in part at least, in the ability of the alkaloid to block the entry of two-carbon fragments into the tricarboxylic acid cycle.

MATERIALS AND METHODS

Reagents. Sodium pyruvate was prepared from redistilled pyruvic acid by the method of Robertson (1942) and recrystallized until pure, as determined iodometrically (Wieland, 1924). Adenosine triphosphate (ATP) (L. Light and Co.) was freshly weighed for each experiment. Diphosphopyridine nucleotide (DPN) was prepared from fresh baker's yeast as described by LePage (1949). Other chemicals were of either B.P. or A.R. quality.

Homogenates. Glass homogenizers of the Potter and Elvehjem type were used, the tubes being cooled by immersion in a freezing mixture.

Yeast. Torulopsis utilis, var. *major* (National Collection of Type Cultures, no. 6593).

Spectrophotometric determinations. A Unicam Quartz spectrophotometer was used.

EXPERIMENTAL AND RESULTS

Effects of cocaine on respiration of Torulopsis utilis

Preliminary experiments showed that cocaine (0·004 M) inhibited the respiration of *T. utilis* with glucose as substrate by about 25%, with pyruvate as substrate by about 80%; that addition of vitamin B_1 did not modify the effects of cocaine and that, unlike the effects on *Saccharomyces* fermenta-

Table 1. *Effects of cocaine on pyruvate and acetate oxidation by* Torulopsis utilis

Flasks, each containing in a total volume of 3·0 ml. (pH 7·0), yeast suspension (1·0 ml., approx. 5 mg. dry wt.), potassium phosphate buffer (0·05 M), either sodium acetate or sodium pyruvate (0·05 M), with and without cocaine (0·004 M), were shaken at 30° in the Warburg apparatus. O_2 uptakes were measured at intervals during 2–3 hr. The centre wells contained 20% (w/v) KOH (0·15 ml.). Gas phase, air. The figures quoted are the means of duplicates which agreed within 5%.

	Acetate			Pyruvate		
	O_2 consumed (μl.)			O_2 consumed (μl.)		
Period	Absence of cocaine	Cocaine (0·004 M)	Inhibition (%)	Absence of cocaine	Cocaine (0·004 M)	Inhibition (%)
1st 30 min.	193	167	14	162	136	16
2nd 30 min.	227	147	35	158	103	35
3rd 30 min.	343	150	56	248	106	57
4th 30 min.	376	109	71	280	85	70
5th 30 min.	375	99	74	310	79	75

tion (Ryman & Walsh, 1952), previous growth of the yeast in the presence of cocaine did not appreciably influence these results. Experiments with rat-brain homogenate showed that pyruvate oxidation in brain is inhibited by cocaine to a similar extent.

T. utilis, which readily metabolizes added acetate, was used to compare the effect of cocaine on acetate oxidation with that on pyruvate oxidation by the procedure described in Table 1.

The results (Table 1) show that the inhibition of respiration by cocaine at pH 7 with acetate as substrate parallels that with pyruvate, which suggests that either cocaine inhibits equally the two enzymes for which pyruvate and acetate are the respective substrates or, more probably, that the site of inhibition is at a later stage in the metabolic pathway which is common to both these substances.

Effect of cocaine on citrate synthesis

The effect of cocaine on the synthesis of citrate from acetate by baker's yeast in the presence of a high concentration of magnesium ions, an aconitase inhibitor (Weinhouse & Millington, 1947), has been investigated as follows.

Fresh baker's yeast (5 g.) in distilled water (100 ml.) was aerated at room temperature for 24 hr. The suspension was then mixed with an equal volume of 0·4 M magnesium acetate and the pH adjusted to 7·0 with $NaHCO_3$ or acetic acid. Equal volumes of the mixture, one of which contained cocaine, were then aerated at 30°. Since the pH of the mixtures tended to rise, especially during the first hour, the pH values were readjusted at intervals during the experiment.

Samples (2·0 ml.) were withdrawn, at zero time and at intervals, and mixed with 30% (w/v) trichloroacetic acid (1·0 ml.). The mixtures were heated to nearly boiling, centrifuged and the clarified solutions (1·0 ml.) were used for determination of citrate by the method of Natelson, Pincus & Lugovoy (1948).

The results, expressed as percentage inhibition (Fig. 1), are calculated from the amounts of citrate formed during the second and third hours when the rate of synthesis is maximum (approx. 100 μg./ml. yeast suspension/hr.), while the pH shows less tendency to change and the cocaine has had time to penetrate the cells.

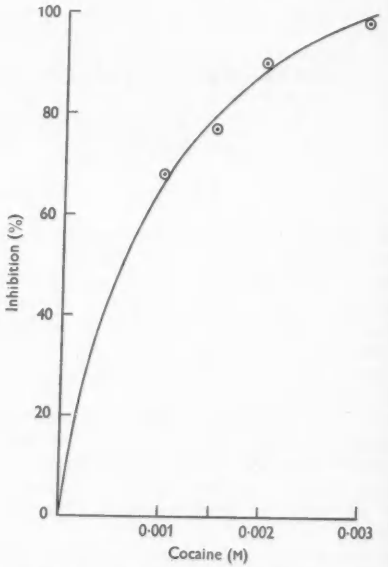


Fig. 1. Inhibition of citrate synthesis from acetate by baker's yeast in the presence of cocaine. For conditions see the text.

In a preliminary report that cocaine inhibits citrate synthesis, the analytical data from one typical experiment have been published (Ryman & Walsh, 1953).

In order to be sure that the inhibitory action of cocaine on citrate synthesis was not peculiar to yeast, an experiment was carried out using brain homogenate with pyruvate and oxaloacetate as substrates. Cocaine (0·002 M) completely inhibited the synthesis of citrate in this system.

Effect of cocaine on oxidative decarboxylation of pyruvate

The effect of cocaine on the degradation of pyruvate to acetate by dialysed pigeon-brain homogenate (Banga, Ochoa & Peters, 1939) was determined at 37° and pH 7·4. The components of the system were the same as those employed by Coxon, Liébecq & Peters (1949). Acetate present, at zero time and after 1 hr., was estimated by the method of Bartley (1953) with the minor modification that sulphuric acid was used in place of hydrochloric and the quantity of 2:4-dinitrophenylhydrazine was increased fourfold. Oxygen uptakes were simultaneously recorded.

The results (Table 2) show that the inhibitory action of cocaine on the step, pyruvate → acetate, is insufficient to account for the effect on the complete oxidation of pyruvate.

For these results we wish to thank Mr A. R. Fahmy, who, while investigating the action of nicotine on pyruvic dehydrogenase, included these experiments with cocaine.

Effect of cocaine on choline acetylase

The effect of cocaine on the anaerobic synthesis of acetylcholine from acetate and choline by a system containing choline acetylase in the presence of ATP has been investigated as follows.

Table 2. *Effect of cocaine on oxidative decarboxylation of pyruvate by a dialysed pigeon-brain homogenate*

Each flask contained ATP, 1 μmole; $MgCl_2$, 4 μmoles; 0·08 M potassium phosphate buffer, pH 7·4; sodium pyruvate, 30 μmoles; brain tissue, approx. 300 mg.; ± cocaine; total vol., 2·5 ml.

Cocaine	Acetate formed (μg./hr.)	O_2 consumed (μl./hr.)	Inhibition (%) of: Acetate formation	Inhibition (%) of: O_2 consumption
Nil	577	132	—	—
0·001 M	532	118	8	11
Nil	510	99	—	—
0·002 M	420	86	18	14

Table 3. *Effect of cocaine on choline acetylase*

The acetylcholine synthesized, in the presence and in the absence of cocaine, from acetate and choline in a choline acetylase system, reconstituted according to Nachmansohn *et al.* (1949), was determined by the method of Hestrin (1949). In experiments 1, 2 and 3 acetylcholine was determined also by the frog rectus method.

Expt.	Acetylcholine (μg./g. dried brain/hr.): Absence of cocaine	Acetylcholine (μg./g. dried brain/hr.): Cocaine (0·002 M)
1	500	400
2	400	400
3	800	660
4	940	980
5	480	440

Choline acetylase was prepared from acetone-dried brain of guinea pig or rabbit and purified by the method of Nachmansohn, Hestrin & Voripaieff (1949). Either a boiled extract from washed brewer's yeast or a solid preparation (Korkes, Campillo, Gunsalus & Ochoa, 1951) from guinea pig or rabbit liver was used as the source of coenzyme A.

Mixtures (5·0 ml.), with and without cocaine, containing enzyme solution (1·0 ml. equivalent to 250 mg. dried brain), yeast extract (3·0 ml.) or crude coenzyme A (10 mg.) and, in final concentrations, KCl (0·04 M), choline (0·02 M), sodium acetate (0·02 M), cysteine (0·02 M), $CaCl_2$ (0·002 M), $MgCl_2$ (0·0002 M), ATP (0·003 M), eserine (0·001 M), were incubated at pH 7·0 and 37° in N_2-filled, stoppered tubes. Samples (2·0 ml.) were withdrawn at zero time and after 1 or 2 hr. for acetylcholine determination by the chemical method of Hestrin (1949) with the following modifications.

Since cocaine, being an ester, reacts with hydroxylamine, and the product gives a colour with $FeCl_3$, it was necessary to extract the cocaine from the samples by shaking with $CHCl_3$ (3·0 ml.). Cocaine is completely extracted by $CHCl_3$ from the solution at pH 7; eserine, which also interferes, is simultaneously removed.

When extract of yeast was used it was necessary to remove protein by precipitation with trichloroacetic acid before readjusting the pH and extracting with $CHCl_3$.

During the incubation a trace of cocaine is hydrolysed to benzoylecgonine which is not extracted by the $CHCl_3$ but which gives a slight colour with the reagents. In each experiment, therefore, a third tube, containing cocaine and buffer only, was included so that the necessary corrections could be made.

The results (Table 3) suggest that cocaine has little effect on the isolated choline acetylase system and that the much higher inhibition of acetylcholine synthesis (25 % by 0·001 M cocaine), which we have observed in a preliminary experiment with respiring fresh brain homogenate, is a secondary effect due to inhibition of respiration.

In some of the experiments, acetylcholine was also determined by the frog rectus method. Cocaine, in the dilutions used, did not interfere and the results were in good agreement with those obtained chemically. We wish to express our thanks to Dr C. C. Mackenzie who carried out the pharmacological determinations.

Effect of cocaine on acetylation of sulphanilamide

The effect of cocaine on the sulphanilamide-acetylating system of liver has been investigated as follows.

Pigeon-liver extracts were prepared from acetone-dried powder as described by Johnson & Quastel (1953). Mixtures (3·5 ml.), with and without cocaine, containing liver extract (1·0 ml. equivalent to 60 mg. acetone powder), sulphanilamide (100 μg.) and, in final concentrations, potassium phosphate buffer (0·017 M), $NaHCO_3$ (0·024 M), $MgCl_2$ (0·004 M), sodium acetate (0·017 M), ATP (0·0025 M), DPN (0·0043 M) were gassed with 7 % CO_2–93 % N_2 and shaken in the Warburg apparatus at 37° and pH 7·5. Sulphanilamide,

present at zero time and after 1·5 hr., was determined by the method of Bratton & Marshall (1939) and the acetylated sulphanilamide calculated by difference.

In four experiments, in which acetylations of up to 50% of the sulphanilamide were obtained, cocaine (0·002M) had no effect, thus confirming our belief that cocaine is not a general inhibitor of all reactions in which 'active acetate' is involved.

DISCUSSION

The hypothesis that the presence of a habit-forming drug might induce adaptive changes in cells whereby the continued presence of the drug became necessary for the metabolic balance of the cells (Ryman & Walsh, 1951) formed the basis for our preliminary experiments with yeasts, in which the behaviour towards cocaine of the normally grown yeast was compared with that of a twin culture grown in the presence of the drug.

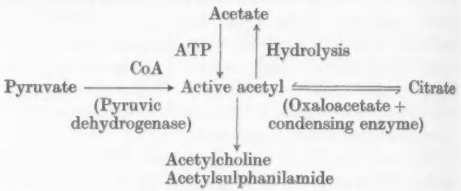
Experiments analogous to those on fermentation (Ryman & Walsh, 1952) have been carried out on respiration, using *T. utilis*. Although the respiration of *T. utilis* is strongly inhibited by cocaine, these experiments have failed to reveal any changes which might contribute to our knowledge of the mechanism of addiction, or to show any protective action of vitamin B_1 against the inhibitory action of cocaine. In view of the possibility that the yeast might have destroyed the cocaine in the growth medium during these experiments, we carried out analyses which showed that 96% of the cocaine originally present in the medium could be recovered after the yeast had been growing in it for a period of 26 hr.

In view of these preliminary experiments, it seemed desirable to discover more precisely the site of action of cocaine in inhibiting cellular respiration. Since, as our experiments have shown, the inhibition of respiration by cocaine is much greater with pyruvate as substrate than it is with glucose, it appeared that the site of inhibition would be found in the metabolic path following the pyruvate stage.

The experiments with *T. utilis* show that cocaine inhibition of respiration with acetate as substrate parallels that with pyruvate. This finding suggests that cocaine acts either by inhibiting the formation of acetyl coenzyme A, whether from acetate or pyruvate, or by inhibiting the further metabolism of the active acetyl group.

The experiments on the effect of cocaine on the biosynthesis of citrate from acetate, in which the further metabolism of citrate is blocked by the presence of a high concentration of magnesium, show that citrate synthesis is inhibited by cocaine more strongly than are any of the other enzyme systems studied. These results indicate that cocaine prevents the entry of acetate into the citric acid cycle and that, in carbohydrate oxidation, the site of inhibition lies between pyruvate and citrate.

It remained, therefore, to investigate the effect of cocaine on the degradation of pyruvate to acetate (pyruvic dehydrogenase system) and on other reactions in which active acetate is either formed or used. The systems under consideration are represented diagrammatically as follows.



The effect of cocaine was studied on the formation of acetate from pyruvate by dialysed brain homogenate, in which the further metabolism of acetate is prevented by lack of cycle components. Since the inhibitions obtained are small in comparison with the effect on citrate synthesis, it would appear that the principal action of cocaine is not to inhibit active acetate formation but to inhibit its utilization.

The results of experiments with the choline-acetylating system of brain and the sulphanilamide-acetylating system of liver show that cocaine does not interfere with these systems to an extent comparable with its effect on the synthesis of citrate. It appears, therefore, that the inhibitory action of cocaine on tissue respiration may be attributed to its ability to block the entry of active acetate into the tricarboxylic acid cycle, and that it is the condensing enzyme which is primarily affected.

It is of interest to consider the results of our enzymic experiments in relation to the biological observations of others. The finding that cocaine inhibits tissue respiration by preventing the oxidation of 'two-carbon fragments' is in complete agreement with the observations of Underhill & Black (1912), who found that daily injections of cocaine into dogs resulted in impaired fat utilization. It has also been observed that, after cocaine injections, unusually large quantities of lactate appear in the urine (Araki, 1891) as one would expect in the event of pyruvate oxidation being retarded. One would also expect some of the symptoms of chronic cocaine poisoning to resemble some of those associated with vitamin B_1 deficiency.

The fact that cocaine does not appreciably inhibit anaerobic synthesis of acetylcholine *in vitro* in the presence of ATP as energy donor does not indicate that acetylcholine synthesis *in vivo* is immune from the inhibitory action of cocaine. On the contrary, experiments with respiring fresh brain homogenate show that acetylcholine synthesis is, under these

conditions, appreciably inhibited, presumably as a result of the block in the essential energy-yielding mechanism of respiration.

The effect of other cocaine-simulating drugs on citrate synthesis is being investigated.

SUMMARY

1. The effects of cocaine on the respiration of yeast and of mammalian tissues with various substrates have been investigated.

2. The effects of cocaine on citrate synthesis, pyruvate degradation and acetylating systems have been investigated. Evidence is presented that cocaine inhibits cellular respiration by blocking the entry of active acetate into the tricarboxylic acid cycle.

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The Effect of Nucleotide and Ischaemic Shock on the Level of Energy-Rich Phosphates in the Tissues

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Shock is best defined, simply, as the general reaction of the body to injury and its most characteristic features in small mammals, such as the rat, are depression of oxygen consumption and body temperature (Tabor & Rosenthal, 1947; Green & Stoner, 1950, 1954). These changes which occur during excessive loss from the carbohydrate stores (Stoner, Threlfall & Green, 1952*a*; Threlfall & Stoner, 1954), imply decreased energy production by the shocked animal and the idea that this is the essential metabolic change after severe injury has been discussed (Green & Stoner, 1954). The possible explanations of the decreased energy production which have been considered may be summarized as follows: (1) Exhaustion of the stored energy of the body—the 'energy depletion' hypothesis of McShan, Potter, Goldman, Shipley & Meyer (1945). (2) 'Uncoupling' of phosphorylation from the oxidative production of energy. (3) Incomplete metabolism of energy-yielding substrate.

Attempts have been made to analyse these possibilities (Stoner *et al.* 1952*a*, *b*; Threlfall & Stoner, 1954) and in the experiments now described the view that the depressed metabolism of the animal in shock is due to exhaustion of its stores of energy-rich compounds has been examined. In the light of current biochemical thought (Lipmann, 1941; Krebs, 1953) this would be represented by depletion of the phosphocreatine (PC) and adenosine triphosphate (ATP) of the tissues which satisfy their immediate energy requirements at the expense of the 'energy-rich bonds' ($\sim$P) of these compounds.

The 'energy-depletion' hypothesis was first put forward by Potter and his co-workers (McShan *et al.* 1945; LePage, 1946*a*, *b*) and was based on the appearance of the breakdown products of carbohydrate and nucleotide metabolism in excessive amounts in the blood of the shocked animal and a fall in the ~P content of the tissues. The most favourable evidence was found when shock was produced by haemorrhage or by the revolving drum described by Noble & Collip (1941), i.e. in animals with generalized tissue damage, but as pointed out by Wilhelmi (1948), the tissue changes were not gross. In shock following more limited tissue injury (hind-limb ischaemia), Bollman & Flock (1944) and Goranson, Hamilton & Haist (1948) did not find gross changes in the tissue nucleotides outside the damaged area although Kovách *et al.* (1952) have recently reported a generalized fall in the tissue ATP content. PC and ATP are, of course, broken down in the ischaemic tissue (Bollman & Flock, 1944; Macfarlane & Spooner, 1946; Bielschowsky, unpublished observations; Goranson *et al.* 1948; Kovách *et al.* 1952).

To investigate the 'energy-depletion' hypothesis more fully, ~P levels in the muscle, brain and liver of rats in both nucleotide and ischaemic shock have been studied. Although nucleotide shock is, in one sense, artificial it closely resembles traumatic shock (Green & Stoner, 1950). The processes of shock are accelerated by a raised environmental temperature (Tabor & Rosenthal, 1947; Green & Stoner, 1950) and advantage was taken of this in order to emphasize any changes in ~P distribution. Nucleotide shock was studied in adrenalectomized rats for the same reason (Stoner & Green, 1950).

METHODS

For experiments on muscle, albino rats (average weight 180 g.) were obtained from the same source as before (Stoner *et al.* 1952*a*, *b*) and fed on Edinburgh rat cube no. 86 (North-Eastern Agricultural Cooperative Society, Aberdeen). For experiments on liver and brain, albino rats of the Porton strain were used, fed on M.R.C. diet 41 (Bruce & Parkes, 1949). In all experiments the animals were fasted overnight before use. The response of the two strains to the shock-inducing procedures was identical. No difference was found in the P distribution in the muscle of the two strains.

Production of shock. Four-hour periods of bilateral hind-limb ischaemia were produced with rubber tourniquets (Rosenthal, 1943). Nucleotide shock was produced by the injection of MgATP (the magnesium salt of adenosine triphosphate), intraperitoneally except in the adrenalectomized rats when it was given intramuscularly. The dose, which varied with the nature of the experiment, is given in the tables. MgATP was prepared from the Ba salt (Boots Pure Drug Co. Ltd.) either as previously described (Green & Stoner, 1950) or by shaking the Ba salt, suspended in water, with Amberlite IR-120 to convert it into the free acid (Osborn, 1953) and then neutralizing it with $Mg(OH)_2$. In a few experiments ATP was obtained as the free acid (L. Light and Co. Ltd.) and neutralized with $Mg(OH)_2$. The purity of the samples was determined chromatographically (Eggleston & Hems, 1952) and only those samples in which the ratio of ATP to ADP was not less than approximately 70/30 were used. The volume of solution used for injection was based on the amount of P liberated by 7 min. hydrolysis in N-HCl at 100°, expressed as ATP. The material did not appear to contain inorganic pyrophosphate.

Adrenalectomy. Adrenalectomy was carried out using the dorsal approach; the rats were under ether anaesthesia and were given 1% (w/v) NaCl to drink after the operation. They were used at the end of 3 days.

Preparation of tissue samples for analysis. Muscle samples were obtained from the hind limb in nucleotide shock and from the fore limb and pectoral muscles in ischaemic shock. Pentobarbitone sodium (Nembutal—Abbott Laboratories Ltd.) and αβ-dihydroxy-γ-(2-methoxyphenoxy)propane (Myanesin—British Drug Houses Ltd.) were used in the controls and experimental animals sampled before death and the extraction of the muscle with 10% (w/v) trichloroacetic acid (TCA) was carried out as described before (Stoner *et al.* 1952*b*).

Samples of liver and brain were obtained from rats frozen in liquid O_2. The controls and the experimental animals sampled before death were anaesthetized with Nembutal (5 mg./100 g. body wt. intraperitoneally) before freezing. When the rat was frozen the head was removed, divided along the sagittal plane with a chisel and the brain removed as completely as possible. The abdomen was broken open and samples of liver removed, avoiding the large blood vessels as far as possible. Brain was ground up in an ice-cold mortar with 6 vol. ice-cold 10% (w/v) TCA and liver was extracted with 6 vol. TCA in a small 'homogenizer' (Folley & Watson, 1948). The protein precipitate was removed by centrifuging in a refrigerated centrifuge as for the muscle extract.

Analytical methods. The muscle extracts were analysed as before (Stoner *et al.* 1952*b*). In this scheme PC-P is the difference between the inorganic P precipitated, as $MgNH_4PO_4$, from the extract before and after standing for 16 hr. at 21°. The further P liberated by 7 min. acid hydrolysis in N-HCl at 100° (Δ 7 min. P) has been taken to represent the two labile phosphate groups of ATP. The contribution of other acid-labile phosphates to this fraction is negligible in muscle (Bendall, 1951). This was confirmed (Table 1) by finding that the Δ 7 min. P was almost exactly twice the P of the terminal phosphate group of ATP estimated by the potato pyrophosphatase method (*vide infra*).

Table 1. *Comparison of methods for the estimation of ATP in muscle*

Δ 7 min. P, determined by hydrolysis with N-HCl and terminal P of ATP, determined by the potato pyrophosphatase method, in rat muscle. Theoretically the Δ 7 min. P should be twice the terminal P of ATP level.

Rat no.	Δ 7 min. P (mg. P/g. wet wt.)	Terminal P of ATP (mg. P/g. wet wt.)
498	0·36	0·19
499	0·44	0·20
550	0·38	0·20
15	0·38	0·18

The total acid-soluble P, inorganic P and PC-P of the brain extracts were determined as for the muscle extracts. The Δ 7 min. P was also estimated, but the contribution from other labile phosphates is too great for this to be equated with the labile groups of ATP (Kerr, 1942; LePage, 1948). The terminal P of ATP was determined, after removing the TCA with ether, by the potato pyrophosphatase method of Lee & Eiler (1951). The enzyme was prepared from mature potatoes and the optimum conditions (temperature, ratio of enzyme to substrate) for the liberation of the terminal P only were determined for each batch of enzyme. The more cumbersome method of Kalckar (1947) for the determination of nucleotides was tried but found unsatisfactory for the reasons given by Munch-Petersen (1953).

The following fractions were determined on liver extracts: total acid-soluble P, inorganic P after 16 hr. at 21° and Δ 7 min. P. No attempt was made to determine any PC present in the liver (Ennor & Rosenberg, 1952). As in brain, phosphate esters other than ATP contribute to the Δ 7 min. P (LePage, 1948) so that the terminal P of ATP was again determined with potato pyrophosphatase.

To obtain confirmation of the origin of the inorganic P liberated from these extracts by potato pyrophosphatase additional tests were carried out on the extracts from six livers and four brains. Good agreement was found between the values for the terminal P of ATP determined with the pyrophosphatase and the values obtained when the adenine nucleotides were absorbed from the extract on to a column of activated charcoal (British Drug Houses Ltd.) followed by elution with 5% (v/v) aqueous pyridine (Crane & Lipmann, 1953) and chromatographic separation of the nucleotides (Eggleston & Hems, 1952).

Plasma inorganic P was determined after precipitation as $MgNH_4PO_4$ (Stoner *et al.* 1952*b*).

In all cases P was determined by the method of Fiske & Subbarow (1925), using either a 'Spekker' absorptiometer (Hilger and Watts, London) or a Coleman 'Junior' spectrophotometer (Coleman Instruments Inc., Maywood, Ill.) for the colour comparison. The results of the tissue determinations are expressed as mg. P/g. wet wt. The water content of the muscle, brain and liver has been taken as 75, 80 and 73% respectively, being the means of several determinations. These values were also used for the shocked animals as it was found that any differences which occurred were too slight to warrant a separate determination in each case. The values shown in the tables are means ± standard error except for the results obtained on the liver and brain in rats dying in nucleotide shock at 30° where the range is shown because of the small number of animals and the high degree of variability. The statistical comparison of the means has been done by Student's '*t*' test.

The P distribution found in normal tissues was in good agreement with that described for the rat by other workers. Because of the significantly lower Δ 7 min. P level in the fore-limb muscle and its lower total acid-soluble and inorganic P content at 20°, muscle from the experimental animals was always compared with muscle removed under similar environmental conditions from the same site in the controls. This principle was also applied to liver and brain, although P distribution there was not significantly affected by environmental temperature. In brain the inorganic P levels were rather higher and the PC-P levels rather lower than those reported by others (LePage, 1946*a*; Richter,

Table 2. *Phosphate distribution in the hind-limb muscles of normal rats at death in nucleotide shock*

Adenosine triphosphate administered intraperitoneally to normal rats and intramuscularly to adrenalectomized rats. Number of observations in each case shown in parentheses.

Treatment	Environmental temperature (°)	Time after injection (average)	Total acid-soluble P	Inorganic P	Phosphocreatine P	Δ 7 min. P
			(mg. P/g. wet wt.)			
Controls	20	—	1·81 ± 0·02 (45)	0·40 ± 0·02 (45)	0·55 ± 0·02 (45)	0·45 ± 0·02 (44)
Controls	30	—	1·81 ± 0·02 (6)	0·41 ± 0·04 (6)	0·63 ± 0·03 (6)	0·48 ± 0·02 (6)
ATP (125–200 mg. Mg ATP/100 g. body wt.)	20	5 hr. 7 min.	1·98 ± 0·02* (23)	0·51 ± 0·03* (23)	0·51 ± 0·03 (22)	0·44 ± 0·01 (22)
ATP (150–220 mg. Mg ATP/100 g. body wt.)	30	2 hr. 56 min.	1·93 ± 0·03* (4)	0·51 ± 0·07 (4)	0·59 ± 0·03 (4)	0·49 ± 0·03 (4)
Adrenalectomized controls	20	—	1·77 ± 0·03 (5)	0·37 ± 0·02 (5)	0·52 ± 0·03 (5)	0·48 ± 0·01 (5)
Adrenalectomized + ATP (50–60 mg. Mg ATP/100 g. body wt.)	20	4 hr. 50 min.	1·77 ± 0·05 (5)	0·54 ± 0·05† (5)	0·46 ± 0·06 (5)	0·47 ± 0·01 (5)

* Significantly different from corresponding control mean at $P < 0{\cdot}01$.
† Significantly different from corresponding control mean at $P < 0{\cdot}05$.

Table 3. *Phosphate distribution in the brain of normal rats, rats in nucleotide shock and rats after a 4 hr. period of bilateral hind-limb ischaemia*

Adenosine triphosphate administered intraperitoneally. Number of observations in each case shown in parentheses.

Treatment	(Environmental) temperature (°)	Clinical state	Time after release of tourniquets or injection (average)	Total acid-soluble P	Inorganic P	Phospho-creatine P (mg. P/g. wet wt.)	Δ 7 min. P	Terminal P of ATP
Controls	20	—	—	0·84±0·02 (16)	0·23±0·02 (16)	0·06±0·005 (15)	0·15±0·005 (16)	0·05±0·003 (16)
Controls	30	—	—	0·81±0·02 (10)	0·25±0·02 (10)	0·07±0·01 (12)	0·18±0·02 (11)	0·04±0·003 (11)
ATP (200 mg. Mg ATP/100 g. body wt.)	20	Death	5 hr. 7 min.	0·88±0·08 (6)	0·37±0·08 (6)	0·07±0·02 (6)	0·13±0·02 (6)	0·03±0·004* (6)
ATP (280 mg. Mg ATP/100 g. body wt.)	30	Death	3 hr. 8 min.	0·79–1·09† (3)	0·33–0·61† (3)	0·02–0·09† (3)	0·20–0·59† (3)	0·02–0·03† (3)
Ischaemia	20	Ill	5 hr. 8 min.	0·83±0·03 (9)	0·28±0·04 (9)	0·06±0·01 (9)	0·14±0·01 (9)	0·05±0·01 (9)
Ischaemia	20	Death	9 hr. 47 min.	0·87±0·04 (9)	0·36±0·04 (9)	0·06±0·01 (9)	0·12±0·03 (8)	0·04±0·08 (6)
Ischaemia	30	Ill	34 min.	0·81±0·03 (4)	0·25±0·03 (4)	0·06±0·01 (4)	0·16±0·01 (4)	0·03±0·005 (4)
Ischaemia	30	Death	1 hr. 23 min.	0·89±0·03* (9)	0·46±0·04‡ (9)	N.D. (11)	0·11±0·02‡ (11)	N.D. (7)

* Significantly different from corresponding control mean at $P<0·05$.
‡ Significantly different from corresponding control mean at $P<0·01$.
† Range.
N.D. Not detectable (see text).

Table 4. *Phosphate distribution in the liver of normal rats, rats in nucleotide shock and rats after a 4 hr. period of bilateral hind-limb ischaemia*

Adenosine triphosphate administered intraperitoneally. Number of observations in each case shown in parentheses.

Treatment	Environmental temperature (°)	Clinical state	Time after release of tourniquets or injection (average)	Total acid-soluble P	Inorganic P	Δ 7 min. P (mg. P/g. wet wt.)	Terminal P of ATP
Controls	20	—	—	1·01±0·04 (14)	0·30±0·02 (12)	0·18±0·01 (12)	0·04±0·01 (12)
Controls	30	—	—	0·94±0·05 (8)	0·22±0·01 (8)	0·19±0·01 (8)	0·04±0·004 (7)
ATP (200 mg. Mg ATP/100 g. body wt.)	20	Death	5 hr. 7 min.	1·13±0·08 (6)	0·60±0·03* (6)	0·16±0·03 (6)	0·03±0·004 (6)
ATP (280 mg. Mg ATP/100 g. body wt.)	30	Death	3 hr. 8 min.	1·07–1·26† (3)	0·51–0·58† (3)	0·05–0·08† (3)	N.D.–0·02† (3)
Ischaemia	20	Ill	5 hr. 8 min.	1·11±0·06 (10)	0·44±0·03 (8)	0·12±0·02 (8)	0·04±0·004 (8)
Ischaemia	20	Death	9 hr. 47 min.	1·10±0·04 (9)	0·52±0·01* (9)	0·08±0·01* (9)	0·02±0·004‡ (8)
Ischaemia	30	Ill	34 min.	0·99±0·03 (4)	0·31±0·01* (4)	0·16±0·01* (4)	0·03±0·005 (4)
Ischaemia	30	Death	1 hr. 23 min.	0·95±0·03 (11)	0·47±0·01* (11)	0·08±0·01* (11)	N.D. (6)

* Significantly different from corresponding control mean at $P<0·01$.
† Range.

1952), probably because the rats were larger than the size (40 g.) recommended by Richter (1952) and because of the short period of anaesthesia used. These departures from the optimum conditions were imposed by the nature of the experiments.

RESULTS

Phosphate distribution in nucleotide shock

The phosphate levels in the muscle of normal and adrenalectomized rats in fatal nucleotide shock are shown in Table 2. The changes in the total acid-soluble and inorganic P fractions may reflect extracellular changes as the plasma inorganic P in the ATP-treated rats, at 20°, was very high; $56{\cdot}7 \pm 0{\cdot}27$ mg./100 ml. (controls, $6{\cdot}50 \pm 0{\cdot}27$). The effect of nucleotide shock on P distribution in the brain and liver is shown in Tables 3 and 4.

Phosphate distribution in ischaemic shock

The effect of hind-limb ischaemia on phosphate distribution in the fore-limb muscle is shown in Table 5. The apparently progressive changes in the total acid-soluble P, inorganic P and PC-P levels could only be proved statistically in the case of inorganic P where the difference between the level in the ill animals and those at death was significant at $P < 0{\cdot}01$.

The mean values for the brain (Table 3) suggest that at 20° phosphate distribution was unaltered by limb ischaemia. The true picture is obscured by pooling the results in this way. Certainly no change was found in the ill rats examined before death, but those which died fell into two groups, those dying within 5 hr. 10 min. of removing the tourniquets and those which were in shock much longer and survived for from 12 hr. 45 min. to 21 hr. The different chemical findings in these two groups are shown in Table 6.

In all but two of the rats dying in ischaemic shock at 30° (Table 3) the PC and ATP levels were below the threshold for the methods used; and in these two animals the PC-P levels were 0·04 and 0·06 mg. P/g. wet wt. and the level of the terminal P of ATP was 0·03 mg. P/g. wet wt.

The liver changes are shown in Table 4 and their correlation with survival time at 20° in Table 6. This latter is not as obvious as in the brain but there is indication that a similar process is at work. Again ATP could only be detected in two rats dying at 30° where terminal P levels of 0·01 and 0·02 mg. P/g. wet wt. were found.

DISCUSSION

The only evidence of 'energy-depletion' in the muscle in shock is the progressive fall in PC-P in the undamaged muscle in ischaemic shock. These results resemble those of Bollman & Flock (1944). The fall

Table 5. *Phosphate distribution in the fore-limb and pectoral muscles of normal rats and rats after a 4 hr. period of bilateral hind-limb ischaemia*

Number of observations in each case shown in parentheses.

Treatment	Environmental temperature (°)	Clinical state	Time after release of tourniquets (average)	Total acid-soluble P	Inorganic P	Phospho-creatine P	Δ 7 min. P
				(mg. P/g. wet wt.)			
Controls	20	—	—	1·63 ± 0·03 (10)	0·34 ± 0·02 (10)	0·57 ± 0·03 (10)	0·40 ± 0·02 (10)
Controls	30	—	—	1·89 ± 0·05 (22)	0·47 ± 0·02 (18)	0·54 ± 0·03 (17)	0·42 ± 0·01 (22)
Ischaemia	20	Ill	3 hr. 30 min.	1·77 ± 0·03 (7)	0·43 ± 0·02* (7)	0·50 ± 0·01 (7)	0·42 ± 0·02 (7)
Ischaemia	20	Death	6 hr. 15 min.	1·79 ± 0·02† (5)	0·58 ± 0·04* (5)	0·40 ± 0·03* (5)	0·42 ± 0·01 (5)
Ischaemia	30	Ill	34 min.	1·81 ± 0·03 (12)	0·58 ± 0·05† (10)	0·41 ± 0·04 (9)	0·39 ± 0·02 (12)
Ischaemia	30	Death	1 hr. 23 min.	1·93 ± 0·06 (16)	0·73 ± 0·02* (15)	0·28 ± 0·04* (15)	0·44 ± 0·02 (16)

* Significantly different from corresponding control mean at $P < 0{\cdot}01$.
† Significantly different from corresponding control mean at $P < 0{\cdot}05$.

* Significantly different from corresponding control mean at $P<0{\cdot}01$.
† Range.

Table 6. *The concentrations of Δ7 min. P and P liberated from ATP by potato pyrophosphatase in the brain and liver of rats at death related to their survival time after a 4 hr. period of bilateral hind-limb ischaemia*

Environmental temperature, 20°. Number of observations in each group shown in parentheses.

	Brain		Liver	
Survival time	Δ 7 min. P (mg. P/g. wet wt.)	Terminal P of ATP (mg. P/g. wet wt.)	Δ 7 min. P (mg. P/g. wet wt.)	Terminal P of ATP (mg. P/g. wet wt.)
2 hr. 25 min.–5 hr. 10 min.	0·04±0·01‡* (4)	0·02±0·008§* (3)	0·06±0·01§* (5)	0·02±0·007 (4)
12 hr. 45 min.–21 hr.	0·19±0·003* (4)	0·05±0·007 (3)	0·11±0·007* (4)	0·02±0·003† (4)
Controls (Tables 3 and 4)	0·15±0·005 (16)	0·05±0·003 (16)	0·18±0·04 (12)	0·04±0·02 (12)

Comparison with control means *=different at $P<0·01$; †=different at $P<0·05$.
Comparison with means of rats with long survival time ‡=different at $P<0·01$; §=different at $P<0·05$.

in PC was not observed by Goranson *et al.* (1948) but they do not seem to have examined their rats at death when it is most pronounced. This change closely resembles that found in diphtheritic toxaemia by Pinchot & Bloom (1950). There was no evidence of the fall in ATP content reported by Kovách *et al.* (1952).

Loss of ~P by muscle is often accompanied by rigidity (Erdös, 1943; Bendall, 1951) but the change is insufficient for that, as the muscles were completely flacid. The chemical differences between the muscles in shock and in death after 'uncoupling' by 3:5-dinitro-*o*-cresol have been mentioned before (Stoner *et al.* 1952*b*) and the changes reported here were less than those found by Parker (1954) after sublethal doses of 3:5-dinitro-*o*-cresol which produced little toxic effect on the rat.

Before attaching much metabolic significance to the fall in PC the possibility that it follows local anoxia due to vascular spasm must be considered. Its occurrence in ischaemic shock and not in nucleotide shock favours this view as distant vascular spasm has been found after the former type of injury (Barnes & Trueta, 1942; Green, Stoner, Whiteley & Eglin, 1951). In unpublished work with Dr M. Bielschowsky on unilateral hind-limb ischaemia in the rat, it was found that changes of this type occurred in the uninjured contralateral limb and these changes could be prevented by denervation of either limb. However, one would hardly expect such a mechanism to be accentuated by a rise in environmental temperature (but see Barcroft, Dornhurst, McClatchey & Tanner, 1952).

In the brain the ~P level only fell when the survival time was short or when the environmental temperature was raised to 30°. Then the fall in ATP content was similar to that reported by LePage (1946*a*) and Kovách *et al.* (1952) but these changes are terminal and may be related to the convulsion which often occurs at death under these conditions. The ~P level of the brain falls in convulsions and anoxia (Klein & Olsen, 1947) and according to Richter (1952), 'as much as 50% of the total brain phosphocreatine was lost in brains fixed in liquid air after 1 sec. of electrical stimulation'. Consequently, the changes observed hardly support an 'energy-depletion' hypothesis, but it must be admitted that functional change can occur in the brain before detectable chemical change (Klein & Olsen, 1947) and that studies on whole brain cannot exclude the possibility of 'energy depletion' in a few vital cells.

In nucleotide shock the ATP level in the liver was only reduced when shock was aggravated by raising the environmental temperature. In ischaemic shock the liver ATP level was low at death at both environmental temperatures but the fall was more striking at 30°. Even then it appeared to be a terminal event. The fall was similar to that found by Kovách *et al.* (1952) but it is difficult to say what effect it would have on hepatic function. It is also impossible to say how far these changes follow alterations in the blood supply to the liver. In haemorrhagic shock there are profound changes in hepatic circulation (Engel, Harrison & Long, 1944) and ~P stores (LePage, 1946*b*) and the survival time can be prolonged by preventing anoxic liver damage (Delorme, 1951). Less is known about the hepatic circulation in ischaemic shock and this is being studied.

The ~P level in the heart has not been studied because the technical difficulties involved (Pollack, Flock & Bollman, 1934; Davies, Francis & Stoner, 1947) made it unlikely that accurate results could be obtained under the present experimental conditions. However, when a rat dies in shock, respiration ceases first and the heart continues to beat slowly, but regularly, and would seem to have sufficient energy for its diminished requirements. The ATP content of the heart is said to be increased in ischaemic shock (Denson, Gray & Jensen, 1953)

but a 48 % reduction was reported by Kovách *et al.* (1952)—probably insufficient to cause cardiac arrest (Chang, 1938).

A simple 'energy-depletion' hypothesis will not account for the appearance of the animal after injury. A rat can obviously exist for many hours in severe shock without a generalized progressive fall in ~P. Such changes as occur may, perhaps, be interpreted as follows:

At 20°, shock, in the rat, is accompanied by a fall in oxygen consumption and body temperature, interpreted as representing a decrease in energy production (Tabor & Rosenthal, 1947). With the fall in energy production the lower body temperature will decrease the need for energy and in this way some sort of balance between ~P production and utilization can be achieved which permits the animal to persist in a state of shock for a long time. Clearly (Table 6) some rats die before this balance is attained, but the biochemical cause of death in both these rats and those which survive much longer is not very apparent.

Raising the environmental temperature to 30° decreases the consumption of oxygen by the normal rat (Benedict & MacLeod, 1929), but by maintaining the body temperature of the shocked rat, it prevents the gross fall in oxygen consumption normally seen in shock (Tabor, personal communication). While energy production may not be so severely depressed, the higher temperature also preserves the demand for energy and, it would seem, to a disproportionate extent for the survival time is shortened and the fall in ~P is exaggerated. Under these conditions no balance can be achieved and perhaps here the rat may be said to die from 'energy depletion' although the fundamental change is the fall in energy production.

SUMMARY

1. The phosphate distribution in the muscle, brain and liver has been studied in rats in shock produced by the injection of adenosine triphosphate and by bilateral hind-limb ischaemia.

2. The total acid-soluble and inorganic phosphate levels in the muscle were increased in nucleotide and ischaemic shock. The phosphocreatine and adenosine triphosphate concentrations were unaffected in nucleotide shock but the phosphocreatine level was reduced in the undamaged muscle in ischaemic shock especially when the environmental temperature was raised to 30°.

3. In the brain, the adenosine triphosphate concentration was reduced at death in nucleotide shock. In ischaemic shock this change was only seen, under ordinary environmental conditions, at death when the survival time was short or when the environmental temperature was increased to 30°. Under the latter conditions there was an increase in the total acid-soluble and inorganic phosphate levels and a pronounced fall in the phosphocreatine content. For the most part the changes in the acid-labile phosphate fraction (Δ 7 min. P) followed those in the adenosine triphosphate fraction except that this fraction was increased if the rat survived for a long time at an environmental temperature of 20°.

4. In the liver an increase in the inorganic phosphate was the only significant change in nucleotide shock at 20°, but at 30° there was also a reduction in the labile phosphate and adenosine triphosphate levels. In ischaemic shock these changes were seen at both environmental temperatures.

5. These results are discussed from the point of view of the 'energy depletion' hypothesis which has been advanced to explain the state of the animal after severe injury. It is concluded that this hypothesis can only be applied to death under certain conditions and that the results indicate a fundamental decrease in energy production.

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Leuco-Anthocyanins

1. DETECTION AND IDENTIFICATION OF ANTHOCYANIDINS FORMED FROM LEUCO-ANTHOCYANINS IN PLANT TISSUES

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A method for the detection of leuco-anthocyanins, and the chromatographic identification of the anthocyanidins formed from them by boiling with mineral acid, has been described in an earlier paper (Bate-Smith, 1953). This method has been improved and applied to the leaves and other tissues of numerous species of plants. In the present paper the results of identification of anthocyanidins produced from leuco-anthocyanins in some of these species are reported. In the paper which follows the systematic distribution of the leuco-anthocyanin reaction in leaves is discussed.

EXPERIMENTAL AND RESULTS

Paper chromatographic separation of anthocyanidins

The main problem is to obtain a solution of the anthocyanidins in sufficient concentration for paper chromatography, without at the same time concentrating irrelevant substances. This is accomplished by converting the leuco-anthocyanins into anthocyanidins in aqueous solution (rather than in methanol as described previously) and extracting the anthocyanidins with *iso*amyl alcohol.

Conveniently, 0·2–1·0 g. of tissue is covered with 2N-HCl (about 3 ml.) in a test tube, and heated in boiling water for 20 min. The aqueous solution is decanted (filtered if necessary) into a small narrow test tube and shaken with sufficient *iso*amyl alcohol (3-methylbutan-1-ol) to give a supernatant layer just deep enough to be drawn cleanly into a capillary tube, from which the solution is spotted on the starting line of the chromatogram. The applications are repeated, employing a current of hot air to accelerate drying, until the colour is deep enough to ensure visibility of the anthocyanidins on the developed chromatogram. A marker of known identity is applied on each paper.

In order to prevent the anthocyanidins from fading, it is necessary to maintain a low pH during chromatography. This was originally achieved (Bate-Smith & Westall, 1950) by using the upper phase of the mixture *n*-butanol–2N-HCl (1:1, v/v). A solvent brought to our notice by Forestal Laboratories has given better-defined spots and more consistent results. This 'Forestal solvent' consists of water–acetic acid–conc. HCl (10:30:3, v/v). Solvents containing *m*-cresol and HCl have also been tested, and one of these consisting of *m*-cresol–5·5N-HCl–acetic acid (1:1:1, v/v) (acetic acid is added in order to bring the aqueous and phenolic constituents into a single phase) has given promising results. As was found with the phenolic solvents employed in earlier work on the chromatography of flavonoid compounds (Bate-Smith, 1949), this solvent has the effect of suppressing the lyophilic properties of methoxyl groups to a greater extent than the aliphatic solvents, so that the order

Table 1. *$E_{max.}$ and R_F values of some anthocyanidins*

	$E_{max.}$ (mμ.)	Solvent system: *n*-Butanol–HCl R_F value	Acetic acid–HCl R_F value	*m*-Cresol–HCl–acetic acid R_F value
Pelargonidin	530	0·80	0·68	0·82
Cyanidin	545	0·69	0·50	0·69
Paeonidin		0·72	0·63	0·87
Delphinidin	555	0·35	0·30	0·52
Petunidin		0·45	0·45	0·75
Malvidin		0·53	0·60	0·90

Table 2. *Spectrographic examination of anthocyanidins from leaves*

Species	$E_{max.}$ (mμ.)	Chromatographic identification
Cinnamomum sericeum	544	Cyanidin
Eriobotrya japonica	543	Cyanidin
Persea gratissima	545	Cyanidin
Raphiolepis indica	543	Cyanidin
Rhoicissus rhomboidea	547, 553	Cyanidin, delphinidin
Rumex lunaria	545	Cyanidin
Thea sinensis (dried green leaf, China type clone)	543, 557	Cyanidin, delphinidin

of the R_F values of the anthocyanidins is different in this solvent from those in acetic acid–HCl and *n*-butanol–HCl (Table 1).

Most of the results recorded below have been obtained with the Forestal solvent. In every instance so far tested, however, the chromatographic results with the other solvents have agreed with those obtained with the Forestal solvent. Valuable further confirmation that the substances produced are, in fact, anthocyanidins lies in the observation that in all solvents other than those containing strong acid the substances are decolorized.

Spectrographic examination

Anthocyanidins have well-defined peaks in the visible region, either in ethanolic HCl solution or when examined directly as a spot on a paper chromatogram by the method of Bradfield & Flood (1953). By the latter method the values are accurate to no more than ± 2 mμ. Values for six anthocyanidins are given in Table 1.

The anthocyanidin spots from digests of leaves were examined spectrographically on paper chromatograms and gave values of $E_{max.}$ shown in Table 2. It will be seen that the spectrographic data in every case confirmed the chromatographic identification. In the case of leuco-anthocyanin prepared from wood of *Pinus maritima*, and supplied by Professor J. Masquelier, the anthocyanidin having the R_F value of cyanidin gave a maximum of 545 mμ.

Application to various plant tissues

The earlier work (Bate-Smith, 1953) was confined to a study of white flowers, in which interference from other substances present is reduced to a minimum. No difficulty has been experienced in extending the study to leaves, in spite of the presence of chlorophyll, since this is largely destroyed in the process of heating with HCl. Any traces of chlorophyll which may remain are extracted by the *iso*amyl alcohol, and appear on the chromatogram near the solvent front. Leaves and other tissues pigmented with anthocyanin are, however, not easy to work with and have so far been avoided. The leaf tissues examined were from mature leaves not evidently pigmented with anthocyanins, freed from petioles and larger veins. Both immature and senescent leaves may contain anthocyanin pigment which may not be visible on mere inspection, and were therefore avoided in a survey confined to the incidence of leuco-anthocyanins. If anthocyanin pigmentation is not excessive, leuco-anthocyanins might still be detected in such instances by comparison with a control prepared from an aqueous extract of the tissue hydrolysed with dilute acid. Robinson & Robinson (1933) suggested such a procedure, selectively extracting the hydrolysed anthocyanidin with *iso*amyl alcohol and developing the leuco-anthocyanin in the underlying aqueous layer. This would of course only apply if the leuco-anthocyanin were itself insoluble in *iso*amyl alcohol. Rarely, tissues may contain preformed anthocyanin in colourless form which is converted into the normal coloured form on acidification; these also have been excluded from the present study.

Catechins, when heated with hydrochloric acid, are converted into 'phlobaphenes' which dissolve in *iso*amyl alcohol to give deep golden (catechins proper) or brown (gallocatechins) solutions.

The tissues of species belonging to certain families give strong colour reactions which might interfere with the detection and identification of anthocyanidins. Such species are *Caryopteris tangutica* and *Tectona grandis* in Verbenaceae,

Table 3. *Anthocyanidins formed from leuco-anthocyanins in leaves*

The following results record the detection, by paper chromatography, of cyanidin (C) and delphinidin (D) formed from leuco-anthocyanins by treatment with hydrochloric acid (see text). Where the concentration of anthocyanidin was appreciably different from the average this is indicated by the letters *s* (strong) and *w* (weak).

Dicotyledons

Actinidiaceae, *Actinidia chinensis*, C; Aizoaceae, *Carpobrotus acinaciformis*, C; Anacardiaceae, *Cotinus coggygria*, C, D; Anonaceae, *Asimina triloba*, C; Asclepiadaceae, *Periploca graeca*, C; Betulaceae, *Corylus avellana*, D; Caprifoliaceae, *Viburnun lantana*, Cw; Celastraceae, *Euonymus fortunei* 'Silver Queen', C, Ds; *E. japonicus*, C, D; *E. radicans* 'minimus', C, D; Cunoniaceae, *Callicoma serratifolia*, C; Ebenaceae, *Diospyros lotus*, Cw, Dw; Ericaceae, *Arbutus unedo*, C, Dw; *Macleania punctata*, C; *Rhododendron smirnowii*, C; Fagaceae, *Fagus sylvatica*, Cs, D; *F. sylvatica*†, C; Juglandaceae, *Juglans regia*, Cs; Lauraceae, *Cinnamomum sericeum*, C; *Persea gratissima*, C; Leguminosae, *Bauhinia yunnanensis*, C; *Caesalpinia echinata*, D; *C. japonica*, C; *Cercis siliquastrum*, C, D; *Coronilla varia*, C, D; *Derris elliptica*, C; *Desmodium canadense*, Cs, Ds; *Hedysarum multiflorum*, Cw, Ds; *Lespedeza thunbergii*, C, D; *Onobrychis sativa*, C, Ds; *Pithecolobium dulce**, C; *Poinciana regia**, C; *Pterocarpus fraxinifolius*, C; *Rhynchosia phaseoloides*, C, Ds; *Scorpiurus muricatus*, C, D; Limnanthaceae, *Limnanthes douglasii*, C, Ds; Magnoliaceae, *Magnolia denudata* (=*M. conspicua*), Cw; Melastomaceae, *Tibouchina semidecandra*, C, Ds; Moraceae, *Ficus sycomorus*, C; *Humulus lupulus*, C, D; Myricaceae, *Myrica gale*, C, Ds; Oxalidaceae, *Oxalis bupleurifolia*, D; Platanaceae, *Platanus orientalis*, C, Ds; Plumbaginaceae, *Limonium latifolium*, D; *L. sinuatum*, D; Polygonaceae, *Fagopyrum tataricum*, C; *Polygonum aviculare*, D; *Rheum rhaponticum*†, C; *Rumex lunaria*, C; Proteaceae, *Grevillea robusta*, D; Rhamnaceae, *Rhamnus cathartica*, C, D; *R. purshiana*, C, Dw; Rosaceae, *Agrimonia eupatoria**, C; *Chaenomeles speciosa* (=*Cydonia japonica* suct.), C; *Eriobotrya japonica**, C; *Fragaria* (strawberry) 'Auchincruive Climax', C; *Kerria japonica**, C; *Malus sargentii**, C; *Mespilus germanica**, Cs; *Neillia longiracemosa**, C; *Osteomeles schwerinae**, C; *Potentilla fruticosa*, C; *P. montana*, C; *Prunus pissardii*†, C; *Raphiolepis indica**, C; *Sorbaria aitchisonii*, C; *Sorbus aucuparia*, C; *Spiraea salicifolia**, C; Salicacea, *Populus canescens*, C; *Salix elaeagnos* (=*S. incana*), C; Sapindacea, *Koelreuteria paniculata*, C, D; *Xanthoceras sorbifolia*, C, Ds; Saxifragaceae, *Astilbe rosea* 'Queen Alexandra', C; *Boykinia aconitifolia*, C; *Hydrangea hortensis*†, C; *Ribes grossularia*, D; *R. sanguineum* 'splendens', D; *Tellima grandiflora*, C, D; Theaceae *Camellia japonica*, C; *Thea sinensis*, Cw, Dw; Tiliaceae, *Tilia europaea*, C; Ulmaceae, *Ulmus procera*, C; Vitaceae, *Parthenocissus quinquefolia*, C; *Rhoicissus rhomboidea*, C, D; *Vitis vinifera*†, C.

Monocotyledons

Iridaceae, *Iris pseudacorus*, C, Ds; Liliaceae, *Smilax rotundifolia*, C.

Gymnosperms

Ginkgoaceae, *Ginkgo biloba*, D; Gnetaceae, *Ephedra americana*, Ds; *E. nebrodensis*, Ds; Pinaceae, *Araucaria bidwillii*, Cw; D; *Metasequoia glyptostroboides*, C; *Sequoiadendron giganteum*, D; Taxaceae, *Taxus baccata*, C, Dw.

Pteridophyta

Equisetaceae, *Equisetum telmateia*, C; Polypodiaceae, *Blechnum occidentale*, C, D; *Dryopteris elongata*, C, D; *Pellea rotundifolia*, Cw, D; *Pteridium aquilinum*, C; *Pteris childsii*, C.

* Paeonidin also present.
† Results recorded by Robinson & Robinson (1933, 1934).

Table 4. *Anthocyanidins formed from leuco-anthocyanins in fruits, etc.*

Fruits and nuts	Plant part	Antocyanidin
Apple 'Bramley Seedling'	Flesh	Cyanidin
Betel nut	—	Cyanidin
Cacao, Criollo	Bean, cotyledons	Cyanidin
Grape, purple	Skin	Cyanidin (Malvidin and delphinidin preformed)
	Seed	Cyanidin
Groundnut	Testa	Cyanidin
Hazel nut	—	Cyanidin
Pear 'Conference'	Flesh	Cyanidin
Plum 'Victoria'	Skin	Cyanidin
Walnut	Seedcoat	Cyanidin
Roots, etc.		
Bergenia crassifolia	Rhizome	Cyanidin
Fagopyrum esculentum	Root	Cyanidin
*Pinus maritima**	Wood	Cyanidin
Flowers (Bate-Smith, 1953)		
Dombeya spectabilis	White petals	Cyanidin
Erica carnea var. *alba*		Cyanidin
Raphiolepis ovata		Cyanidin

* Material as named and supplied by Professor J. Masquelier, Faculté de Médecine et de Pharmacie, Bordeaux, France.

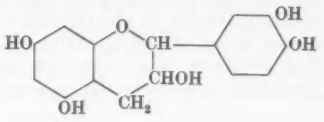
Galium spp. in Rubiaceae, *Escallonia* spp. in Saxifragaceae, *Garrya elliptica* in Garryaceae, *Incarvillea olgae* in Bignoniaceae, and *Aucuba japonica* in Cornaceae. In some instances the substances responsible for these interfering reactions are known (cf. Trim & Hill, 1952).

Sugars in exceptionally high concentration, as in some ripe fruits, when heated with hydrochloric acid give a golden-brown colour, soluble in *iso*amyl alcohol to give a red-brown solution. On the chromatogram a brown zone due to this pigment, which appears cream-coloured when viewed in ultraviolet light, is visible near the solvent front.

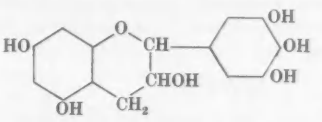
Tentative identifications, based on measurements of R_F values, of the anthocyanidins formed from leuco-anthocyanins present in leaves, seeds and fruits, are recorded in Tables 3 and 4.

DISCUSSION

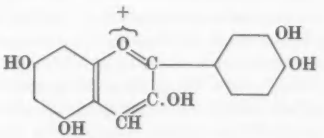
A remarkable feature of the results obtained is that, with only a few exceptions, the leuco-anthocyanins present in the tissues examined yielded only cyanidin and delphinidin when digested with hydrochloric acid. The exceptions were confined to Rosaceae and Leguminosae, where, occasionally,



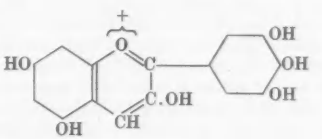
(I)



(II)



(III)



(IV)

an anthocyanidin corresponding in R_F value with paeonidin was present on the chromatogram. These areas on the chromatogram were usually confused with a pinkish brown trail, which occurred in most species of Rosaceae, and also in those of *Fagopyrum*, *Vitis*, *Fagus*, *Populus*, *Boykinia*, *Lespedeza* and *Desmodium*.

The near relationship, from the chemical point of view, of the leuco-anthocyanins and the catechins has been discussed previously (Robinson & Robinson, 1933; Bate-Smith, 1953). Up to the present no hydroxyflavans other than catechin (I) and gallocatechin (II) have been found in nature (cyanomaclurin, occurring uniquely in *Artocarpus integrifolia*, is excepted). Their pattern of hydroxylation is the same as that found in cyanidin (III) and delphinidin (IV), respectively, and it is therefore a matter of some importance, in connexion with the origin and function in the plant of the leuco-anthocyanins and catechins, that the leuco-anthocyanins should be restricted to the patterns of hydroxylation found in the catechins. The systematic significance of these results is discussed in the following paper.

SUMMARY

1. The leuco-anthocyanins present in leaves, fruits and other tissues are converted into anthocyanidins by heating the tissues with 2 N aqueous hydrochloric acid. The anthocyanidins are identified by paper chromatography using the three solvent systems, *n*-butanol–2 N hydrochloric acid (1:1, v/v), water–acetic acid–conc. hydrochloric acid (10:30:3, v/v), and *m*-cresol–acetic acid–5·5 N hydrochloric acid (1:1:1, v/v).

2. Except in Rosaceae and a few Leguminosae (which appear to contain leuco-paeonidin) the leuco-anthocyanins appear to be restricted to leuco-cyanidin and leuco-delphinidin.

The author wishes to thank Dr T. White of the Forestal Land, Timber and Railways Company Ltd., Harpenden, Herts, for supplying the details of the acetic acid–HCl solvent, and Dr T. Swain for the spectrographic data incorporated in Tables 1 and 2. The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Leuco-Anthocyanins

2. SYSTEMATIC DISTRIBUTION OF LEUCO-ANTHOCYANINS IN LEAVES

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In Part 1 (preceding paper) a method of demonstrating the presence of leuco-anthocyanins in plant tissues has been described. A modified version of this method, in which paper chromatographic separation of the anthocyanidins is not carried out, has been applied to the leaves of several hundred species of plants. As a confirmatory test, in the great majority of instances the vanillin reaction has been applied to methanolic extracts of the leaves. In extracts of plant tissues, this reaction is given only, so far as is known, by leuco-anthocyanins and catechins, and the latter do not, of course, give anthocyanidins under the conditions of the primary test. In every instance described below, where a specimen giving a positive anthocyanidin test has been examined chromatographically, anthocyanidin has, in fact, been present on the chromatogram.

METHODS

Anthocyanidin test. This is carried out by heating the sample of leaf tissue in 2N-HCl, and extracting the digest with *iso*amyl alcohol, as described in Part 1, except that the extraction can be carried out in the digestion tube in presence of the solid residue, and that the amount of *iso*amyl alcohol need not be so restricted as when the solution is to be used for chromatography. The shade and depth of colour of the *iso*amyl alcoholic layer are noted. Any colour from deep crimson to faintly pinkish is regarded as positive for anthocyanidin (subject to confirmation by the vanillin test or by chromatography) and is scored according to depth (see Table 1).

Vanillin test. A similar sample of fresh leaf tissue is macerated with methanol (about 2 ml.) in a test tube for approx. 1 min. The (usually) bright green extract is decanted into another tube and shaken with light petroleum to remove chlorophyll. (If the tissue is left in contact too long with methanol the phytyl group of chlorophyll will be transesterified by chlorophyllase, when present, with methyl and the chlorophyll is not then so readily extracted from the methanolic layer.) The extraction with light petroleum is repeated until the methanolic layer is no more than faintly green. One-half of the methanolic solution is then transferred to a third tube, and 3–5 drops of conc. HCl are added to each tube; 3–5 drops of a saturated solution of vanillin in ethanol are added to one of them. A positive reaction varies from one in which a deep cherry-red coloration is produced immediately to one which becomes only perceptibly warmer in tint than the control. When the vanillin reaction is negative, both the control and the test samples usually become bluer in tint, because of the development of a reaction between carotenoid compounds and HCl (cf. Bate-Smith, 1953). This reaction is slower in development than the vanillin reaction, and, moreover, it appears to be inhibited in presence of leuco-anthocyanins. When the latter are present, in high concentration, anthocyanidin is slowly developed in the HCl control sample even in the cold; the blue carotenoid reaction is not only suppressed, but is replaced by a scarlet anthocyanidin reaction.

RESULTS

In Tables 1 and 2 are recorded the results of applying the anthocyanidin test to the leaves of over 500 species of plants. The vanillin test was carried out on 448 (86 %) of these plants and only in four instances was there any clear qualitative disagreement between the results of the two tests.

Discussion of Table 1

The results in this table support the earlier conclusion (Bate-Smith, 1953) that leuco-anthocyanins occur much more commonly in the tissues of woody plants than in those of herbaceous plants. This generalization is strikingly brought out by relating the results to the phylogenetic scheme of Hutchinson (1946, 1948), which is based essentially on a division of dicotyledonous plants into two groups, one, Lignosae, predominantly woody, the other, Herbaceae, predominantly herbaceous. In the Lignosae, the only families so far examined of which the members contain no leuco-anthocyanins are Oleaceae, Loganiaceae, Cornaceae, Araliaceae, Greyiaceae,* Myrsinaceae, Linaceae, Erythroxylaceae, Buxaceae and those in the group Thymeleales to Cucurbitales. Most of these are regarded as 'apical' families in their respective phyletic series. Several of the other 'apical' families such as Asclepiadaceae, Caprifoliaceae, Rubiaceae, Rutaceae, Verbenaceae, and Papilionaceae (Hutchinson) contain many members without leuco-anthocyanins. On the other hand, families recognized as relatively primitive—for example, Magnoliaceae, Theaceae,

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* Willis (1948), following Engler and Prantl, places *Greyia* in Melianthaceae.

Dilleniaceae, Anonaceae, Tiliaceae and Rosaceae—regularly contain leuco-anthocyanins, often in great quantity. In the Herbaceae, the only families in which leuco-anthocyanins have been detected are Saxifragaceae (Hutchinson), Polygonaceae, Ficoidaceae (=Aizoaceae E.P.), Oxalidaceae, Limnanthaceae, Balsaminaceae, and Plumbaginaceae.* The first are 'extracted' by Hutchinson as a herbaceous group from the otherwise woody Saxifragaceae (E.P.). Of the remainder only Limnanthaceae and Balsaminaceae are 'wholly herbaceous' (Metcalfe & Chalk, 1950).

The incidence of leuco-anthocyanins within some of those families which give partly positive and partly negative reactions appears at first sight to be quite irrational. On examination, however, factors can be discerned which seem to influence the presence or absence of leuco-anthocyanin in these cases. The best example for detailed consideration is that of Leguminosae. Although more than sixty species of Leguminosae have been examined, this number is still too small to cover this enormous family adequately. Of the three subfamilies, the Papilionatae have been studied most thoroughly, members of all ten of its tribes being represented in the forty-seven species examined.

The Leguminosae are included by Hutchinson in Lignosae, but the Papilionatae are mainly herbaceous. Of the trees (or near-trees) in this subfamily, *Cladrastis*, *Robinia*, *Wistaria*, *Derris*, *Machaerium* and *Pterocarpus* are positive for leuco-anthocyanin, while *Sophora* and *Laburnum* are negative. Even among the herbaceous members, although the great majority are negative, there is one tribe, viz. the Hedysareae, persistently positive for leuco-anthocyanin. Clearly, woodiness is not the only factor determining presence or absence of leuco-anthocyanin. Some assistance in accounting for this unevenness in distribution is given by Dormer's (1946) division of the Papilionatae into 'pulvinate' and 'epulvinate' groups, depending on the presence or absence of a pulvinus at the base of the leaf. The former includes Sophoreae, Dalbergieae, Phaseoleae and parts of Galegeae and Hedysareae, the latter Podalyrieae, Trifolieae, Loteae, and Vicieae, with the remaining subtribes of Galegeae and Hedysareae. The distribution of species positive and negative for leuco-anthocyanin follows this division remarkably closely. *Sophora* in Sophoreae, and four of the Hedysareae are the only examples which fall outside this generalization.

* *Note added* 23 *April* 1954. Since this paper was submitted for publication, several species of *Primula* have been examined, with results as follows (cf. Table 1): *Primula denticulata*, + +; *P. obconica*, −; *P. verticillata*, + + +; *P. vulgaris*, + +. Primulaceae have therefore to be added to the families of Herbaceae which are positive for leuco-anthocyanin.

Much significance must be attached to Dormer's conclusion that the pulvinate condition is the primitive one, and his observation that 'there is a distinct correlation between the presence or absence of a foliar pulvinus and the general habit of the plant. Every tribe, subtribe, or genus mentioned in the epulvinate column of the table is predominantly herbaceous, whereas in the pulvinate series there are several groups which are predominantly or entirely woody. Furthermore, while the pulvinate series contains some dozens of genera of large forest trees, it is exceedngly rare for an epulvinate species to exceed the stature of a shrub....It is difficult to escape the conclusion that the whole metabolism is different in the two groups—in particular the pulvinate forms are characterized by much more pronounced secretory activity.' The important concept here is that a metabolic pattern associated with woody habit may be found in related species which are themselves of herbaceous habit.

We appear to have, therefore, as factors determining the presence or absence of leuco-anthocyanin in a species: (1) the general character and systematic position of the family to which it belongs, and (2) the stage of evolution of the species within the family, especially in relation to its habit, whether arboreal, climbing, shrubby, or herbaceous. A further question which has to be faced is the reality of the grounds on which affinities, sufficient to warrant the circumscription of species within the bounds of a family, are claimed. Many families—the Euphorbiaceae for instance—are notoriously of doubtful homogeneity, and it is not surprising, therefore, to find their members differing markedly in chemical characters.

Bearing these considerations in mind, the situation in such families as Rosaceae, Saxifragaceae (E.P.), Caprifoliaceae, and Rutaceae appears more rational. In these families the incidence of leuco-anthocyanins decreases as the habit approaches the herbaceous. Leuco-anthocyanins may be absent from shrubs and woody climbers of some tribes, but may nevertheless be present in herbaceous forms of other tribes of the family. As in Moraceae, they may be absent from trees in one division, yet present in herbs of another division of a family; especially if the divisions are so divergent in character as to be treated by some authors as separate families. Essentially, the association of leuco-anthocyanins with woody character underlies the pattern of their distribution in these families. At the same time, and perhaps not altogether independently, there is a tendency for leuco-anthocyanins to be present in the more primitive, and absent from the more advanced, members of a phyletic series. If this criterion were the only one which applied, the Herbaceae as a whole would qualify to be regarded as phylogenetically advanced beyond the Lignosae.

Table 1. *Anthocyanidin reaction in leaves of dicotyledonous plants*

The conventions used in recording the strength of the reaction are: + + + +, exceedingly strong; + + +, very strong; + +, strong; +, moderate; (+), weak; ±, uncertain; −, negative; ×, reaction obscured by development of interfering coloration. In each major subdivision the families are arranged in alphabetical order. Willis (1948) has been used as a guide to the further subdivision of families marked with an asterisk.

Acanthaceae, *Asystasia bella*, −; Aceraceae, *Acer laxiflorum*, + +; *A. negundo* v. *auratum*, (+); *Dipteronia sinensis*, + + +; Actinidiaceae, *Actinidia chinensis*, + +; Aizoaceae, *Carpobrotus acinaciformis*, +; *Tetragonia expansa*, −; *Vanzijlia angustipetala*, −; Amaranthaceae, *Amaranthus cordatus*, −; Anacardiaceae, *Cotinus americanus*, + +; *Mangifera indica*, +; *Cotinus coggygria*, + +; *Rhus illinoensis*, + +; *R. typhina*, ±; Anonaceae, *Asimina triloba*, + +; Apocynaceae, *Acokanthera venenata*, −; *Allamanda neriifolia*, −; *Apocynum androsaemifolium*, + +; *Landolphia florida*†, ×; *Landolphia klainei*†, ×; *Nerium oleander*, −; *Plumeria bicolor*, (+); *Vinca major*, + +; Aquifoliaceae, *Ilex aquifolium*, −; *I. integra*, −; *I. latifolia*, (+); *I. paraguayensis*, (+); Araliaceae, *Hedera helix*, −; Aristolochiaceae, *Aristolochia clematitis*, −; Asclepiadaceae, *Asclepias incarnata*, −; *Periploca graeca*, +; *Vincetoxicum fuscatum*, −; Balsaminaceae, *Impatiens noli-tangere*, + +; *I. parviflora*, + +; Berberidaceae, *Mahonia aquifolium*, −; Betulaceae, *Alnus glutinosa*, +; *Corylus avellana*, + + +; Bignoniaceae, *Bignonia capreolata*, −; *Campsis radicans*, −; *Catalpa bignonioides*‡, ×; *Glaziova bauhinioides*, −; *Incarvillea olgae*§, ×; Bixaceae, *Bixa orellana*, +; Bombacaceae, *Bombax malabarica*, −; *Ceiba pentandra*, +; Boraginaceae, *Nonnea lutea*, ±; Burseraceae, *Bursera* sp., + +; Buxaceae, *Buxus sempervirens*, −; *B. sinensis*, −; *Pachysandra terminalis*, −; Cactaceae, *Zygocactus truncatus*, −; Calycanthaceae, *Chimonanthus praecox*, −; Campanulaceae, *Campanula muralis*, −; *Platycodon grandiflorum*, −; *Specularia hybrida*, −; Caprifoliaceae, *Abelia grandiflora*, +; *Diervilla floribunda*, + +; *D. venosa*, + +; *Dipelta floribunda*, + +; *Lonicera alpigena*, −; *L. glaucescens*, −; *L. nitida*, −; *L. periclymenum*, −; *L. pileata*, −; *L. tatarica*, −; *Sambucus ebulus*, −; *S. nigra*, −; *Viburnum davidii*, + +; *V. lantana*, +; *V. tomentosum* var. *plicatum*, +; Caryophyllaceae‖, *Acanthophyllum spinosum*, −; *Arenaria grandiflora*, (+); *Cerastium biebersteinii*, −; *Dianthus barbatus*, −; *Dianthus* 'Border carnation', −; *Dianthus* 'Cantab', −; *Herniaria ciliata*, −; *Paronychia capella*, −; *Saponaria officinalis*, ×; *Silene fimbriata*, −; *Spergula arvensis*, −; *Stellaria media*, −; Casuarinaceae, *Casuarina glauca*, +; Celastraceae, *Euonymus alatus*, + +; *E. fortunei* 'Silver Queen', + +; *E. japonicus*, +; *E. radicans minimus*, +; Chenopodiaceae, *Atriplex hastata*, −; *Beta maritima*, −; Cistaceae, *Cistus incanus*, + +; *Helianthemum verum* 'Old Gold', (+); Combretaceae, *Terminalia chebula*, +; Compositae, *Baccharis halimifolia*, −; *Cassinia fulvida*, −; *Centaurea cyanus*, −; *Dahlia merckii*, −; *D. variabilis*, −; *Helichrysum bracteatum*, −; *Kleinia cylindrica*, −; *Microglossa albescens*, −; *Olearia gunniana*, −; *O. haastii*, −; *O. macrodonta*, −; *O. virgata*, −; *Senecio fulgens*, −; *Tagetes patula*, −; Convolvulaceae, *Ipomoea learii*, −; Coriariaceae, *Coriaria nepalensis*, −; Cornaceae, *Aucuba japonica*¶, ×; *Cornus amomum*, −; *C. florida*, −; *C. foemina*, −; *C. mas* 'variegata', −; Crassulaceae**, *Bryophyllum tubiflorum*, −; *Sedum aizoon*, ×; *S. middendorffianum*, +; Cruciferae, *Cheiranthus cheiri* (orange)††, ×; *C. cheiri* (yellow), −; *Iberis sempervirens*††, ×; *Isatis glauca*††, × ×; *Lepidium latifolium*, −; *Peltaria alliacea*, −; Cunoniaceae, *Callicoma serratifolia*, + + + +; *Cunonia capensis*, + +; Dilleniaceae, *Candollea tetrandra*, + +; *Hibbertia volubilis*, + +; Dipsacaceae, *Cephalaria ambrosioides*, −; *Scabiosa graminifolia*, −; Ebenaceae, *Diospyros lotus*, +; Elaeagnaceae, *Elaeagnus orientalis*, +; Ericaceae, *Arbutus menziesii*, + +; *A. unedo*, + + +; *Gaultheria cuneata*, + +; *Macleania punctata*, +; *Rhododendron smirnowii*, + + +; *Vaccinium intermedium*, + +; Erythroxylaceae, *Erythroxylum coca*, (+); Eucommiaceae, *Eucommia ulmoides*, + +;

Euphorbiaceae*:

Phyllantheae, *Andrachne colchia*, + +; *Phyllanthus angustifolius*, + +; *Phyllanthus mimosoides*, + +;
Daphniphylleae, *Daphniphyllum macropodium*‡‡, ×;
Acalypheae, *Mercurialis perennis*, −; *Ricinus communis*, −;
Adrianeae, *Manihot utilissima*, ±;
Cluytieae, *Codiaeum angustifolium*, +; *Codiaeum variegatum* var. *aucubifolium*, + +;
Euphorbieae, *Euphorbia aphylla*, −;

Fagaceae, *Fagus sylvatica*, + +; *Quercus macrocarpa*, + +; *Quercus suber*, +; *Quercus warburgii*, +;

Flacourtiaceae*:

Pangieae, *Hydnocarpus wightianus*, + +; *Kiggelaria africana*, −;
Flacourtieae, *Azara gilliesii*, −; *Azara microphylla*, +; *Idesia polycarpa*, −; *Poliothyrsis sinensis*, (+);
Casearieae, *Arechavaletaia uruguayensis*, + +;

Garryaceae, *Garrya elliptica*¶, ×; Gentianaceae, *Menyanthes trifoliata*, −; Geraniaceae, *Erodium cicutarium*, −; *Geranium grandiflorum*††, ×; Guttiferae, *Hypericum androsaemum*, + +; Hamamelidaceae, *Distylium racemosum*, +; *Liquidambar styraciflua*, + +; *Parrotia persica*, +; Hydrophyllaceae, *Hydrophyllum canadense*, −; *Phacelia grandiflora*, −; *P. tanacetifolia*, −; Icacinaceae, *Pennantia cunninghamii*, + +; Juglandaceae, *Juglans regia*, + + +; Labiatae, *Origanum vulgare*, −; *Prostanthera ovalifolia*, −; *Salvia verbenaca*, −; *Teucrium scorodonium*, −; Lardizabalaceae, *Akebia quinata*, −; Lauraceae, *Lindera benzoin*, +; *Umbellularia californica*, +;

Leguminosae*:

Mimosoideae: Acacieae, *Acacia alata*, + +; *A. armata*, +; *A. catechu*, (+); *A. decurrens*, −; *A. retinodes*§§, ×; *A. robusta*, + +; Eumimoseae, *Mimosa pudica*, + +;
Caesalpinioideae: Bauhinieae, *Bauhinia yunnanensis*, +; *Cercis siliquastrum*, +; Cassieae, *Cassia tomentosa*, −; *Ceratonia siliqua*, + +; Eucaesalpinieae, *Caesalpinia echinata*, + +; *C. gilliesii*, −; *C. japonica*, + +; *Gleditschia sinensis*, −; *G. triacanthos*, +;
Papilionatae: Sophoreae, *Cladrastis tinctoria*, +; *Sophora japonica*, −; *S. macrocarpa*, −; Podalyrieae, *Baptisia australis*, −; *Chorizema cordatum*, −; *C. ilicifolium*, −; *Piptanthus nepalensis*, −; *Thermopsis montana*, −; Genisteae, *Laburnocytisus adamii*, −; *L. vulgare*, −; *Petteria ramentaceae*, −; *Spartium junceum*, −; Trifolieae, *Medicago lupulina*, −; *M. sativa*, −; *Melilotus lutea*, −; *Ononis fruticosa*, −; *O. repens*, ±; *Trifolium dubium*, (+); *T. incarnatum*, −; *T. pratense*, −; Loteae, *Anthyllis vulneraria*, −; Galegeae, *Amorpha fruticosa*, + +; *Caragana frutescens*, ±; *Clianthus puniceus*, −; *Colutea arborescens*, ±; *C. orientalis*, ±; *Galega officinalis*, −; *Psoralea macrostachys*, (+); *Robinia pseudacacia*, + +; *Wistaria sinensis*, + +; Hedysareae, *Arachis hypogea*, (+); *Coronilla varia*, +; *Desmodium canadense*, + +; *Hedysarum multiflorum*, + +; *Hippocrepis comosa*, −; *Lespedeza thunbergii*, +; *Onobrychis sativa*, + + +; *Scorpiurus muricatus*, +; Dalbergieae, *Derris elliptica*, + +; *Machaerium firmum*, + +; *Pterocarpus fraxinifolius*, +; Vicieae, *Lathyrus luteus*, −; *L. venatus*, −; *Vicia dumetorum*, −; *V. faba*, −; Phaseoleae, *Glycine soja*, ±; *Phaseolus vulgaris*, −; *Rhyncosia phaseoloides*, + +;

Limnanthaceae, *Limnanthes douglasii*, + + +; Linaceae, *Linum grandiflorum*, −; *L. lewisii*, −; *L. perenne*, −; *Reinwardtia tetragyna*, −; Loganiaceae, *Buddleia* sp., −; *Desfontainia spinosa*, −; Lythraceae, *Lagerstroemia indica*, −; *Lythrum salicaria*, −; *Nesaea salicifolia*, −; Magnoliaceae, *Liriodendron tulipifera*, + +; *Magnolia denudata* (=*M. conspicua*), +; *M. stellata*, −; *M. virginiana*, (+); Malvaceae, *Abutilon* 'Boule de Neige', −; *Abutilon* 'Thompsonii',

Table 1 (*cont.*)

−; *A. vitifolium* var. *album*, +; *Althaea rosea*, −; *Gaya lyallii*, −; *Gossypium herbaceum*, + +; *Hibiscus rosa-sinensis*, −; *H. syriacus*, −; *Pavonia spinifex*, +; Melastomaceae, *Schizocentron elegans*, −; *Tibouchina semidecandra*, + + +; *Tococa* sp., + +; Meliaceae, *Cedrela odorata*, −; *Swietenia mahogoni*, + + +; Melianthaceae, *Greyia sutherlandii*, −;

Moraceae*:

Moroideae, *Dorstenia lujae*, −; *Maclura pomifera*, −; *Morus alba*, −; *M. nigra*, −;

Artocarpoideae, *Artocarpus incisa*, +; *Cudrania javanensis*, −; *C. tricuspidata*, −; *Ficus carica*, −; *F. elastica*, + +; *F. lancifolia*, + +; *F. religiosa*, −; *F. sycomorus*, + +;

Cannaboideae, *Cannabis sativa*, ±; *Humulus lupulus*, + + +;

Myoporaceae, *Myoporum acuminatum*, −; *Oftia africana*††, ×; Myricaceae, *Myrica gale*, + +; Myrsinaceae, *Myrsine africana*, −; Nyctaginaceae, *Bougainvillaea glabra* var. *sullivanii*, −; *Oxybaphus nyctagineus*, −; *Pisonia brunnoniana*††, ×; Nymphaeaceae, *Nymphaea* sp., −; *Victoria regia****, ±; Nyssaceae, *Davidia vilmoriniana*, −; Oleaceae, *Fraxinus excelsior*, −; *Fraxinus mariesii*, −; *Ligustrum vulgare*, −; *Phillyrea latifolia*, −; Oxalidaceae, *Oxalis bupleurifolia*, + +; *O. dispara*, + +; *O. namaquana*, +; *O. pes-caprae*, −; *O. tuberosa*, ±; *O. valdiviensis*, (+); Papaveraceae, *Dendromecon rigidum*, −; Passifloraceae, *Passiflora coerulea*, −; Phytolaccaceae, *Phytolacca americana*, −; Piperaceae, *Piper futokadruna*, −; Pittosporaceae, *Pittosporum crassifolium*, −; Plantaginaceae, *Plantago major*, −; Platanaceae, *Platanus acerifolius*, + +; *P. orientalis*, + +; *P. orientalis* var. *cuneatus*, + +; Plumbaginaceae, *Armeria maritima*, +; *Ceratostigma willmottianum*, +; *Limonium latifolium*, + +; *L. sinuatum* (cultivar), + +; *Plumbago capensis*, + +; Polemoniaceae, *Cobaea scandens*, ±; Polygalaceae, *Polygala chamaebuxus* var. *grandiflora*, −; Polygonaceae, *Atraphaxis hispida*, +; *Fagopyrum esculentum*, +; *F. tataricum*, + +; *Polygonum alpinum*, +; *P. aubertii*, +; *P. aviculare*, +; *P. hydropiper*, + +; *P. persicaria*, +; *Rumex acetosa*, + +; *R. maximus*, + +; *R. patientia*, +; Portulacaceae, *Portulaca grandiflora*, −; *P. oleracea*, −; Proteaceae, *Banksia serrata*, + +; *Grevillea ornithopoda*, + +; *G. robusta*, + +; *G. sulphurea*, +; *Hakea multilineata*, + +; *H. teretifolia*, + +; *H. ulicina*, + +; *Macadamia ternifolia*, + +; Ranunculaceae, *Paeonia humilis*, −; *Zanthorhiza apiifolia*, −; Rhamnaceae, *Ceanothus arnoldii*, + +; *Colletia cruciata*, +; *Paliurus australis*, +; *Rhamnus cathartica*, +; *R. purshiana*, +; Rhizophoraceae, *Rhizophora* sp., + + +;

Rosaceae*:

Spiraeoideae: Spiraceae, *Neillia longiracemosa*, + +; *Spiraea japonica*, +; *S. salicifolia*, + +; Quillajeae, *Exochorda racemosa*, + +;

Pomoideae: *Chaenomeles speciosa* (=*Cydonia japonica*), +; *Cotoneaster horizontalis*, +; *Crataegus crus-galli*, +; *Mespilus germanica*, + + +; *Osteomeles schwerinae*, + + +; *Sorbus aucuparia*, +;

Rosoideae: Kerrieae, *Kerria japonica*, −; Potentilleae, *Fallugia paradoxa*, −; *Fragaria* (cultivated strawberry), + +; *Potentilla alchemilloides*, +; *P. fruticosa*, + +; *P. montana*, +; *Rubus deliciosus*, −; Ulmarieae, *Filipendula ulmaria*, + +; Sanguisorbeae, *Agrimonia eupatoria*, + + +; Roseae, *Rosa bracteata*, (+); *R. damascena*, +; *R. foetida* var. *persiana*, +; *R. multiflora*, + +; *R. rubiginosa*, +; *R. rugosa*, +; *R. spinosissima* 'lutea', +; *R. soulieana*, (+); *R. xanthina*, +;

Prunoideae: *Prunus laurocerasus*, + +; *P. mahaleb*, +;

Rubiaceae*:

Cinchonoideae: Cinchoneae, *Cinchona succirubra*, −; Mussaendeae, *Mussaenda luteola*, + +;

Caffeoideae: Ixoreae, *Coffea arabica*, +; *C. robusta*, + +; *Pavetta natalensis*, + +; Galieae, *Galium aparine*‡‡, ×; *G. verum*††, ×;

Rutaceae*:

Rutoideae: Zanthoxyleae, *Barosma lanceolata*, + +; *Choisya ternata*, −; *Zanthoxylum bungei*, + +; Ruteae, *Dictamnus fraxinella*, −;

Toddalioideae: *Ptelea trifoliata*, −; *Skimmia japonica*, −;

Aurantioideae: *Aegle spinosa* (=*Ponciras trifoliata*), −;

Salicaceae, *Populus canescens*, + +; *P. deltoides*, +; *Salix acutifolia*, +; *S. babylonica*, + +; *S. caprea*, +; *S. elaeagnos* (=*S. incana*), + +; Santalaceae, *Santalum album*, −; Sapindaceae, *Koelreuteria paniculata*, + +; *Nephelium longana*, + +; *Sapindus saponaria*, −; *Xanthoceras sorbifolia*, + +;

Saxifragaceae*:

Saxifragoideae, *Astilbe rosea*, + +; *Bergenia crassifolia*, + +; *Boykinia aconitifolia*, + + +; *Tellima grandiflora*, + +;

Francoideae, *Francoa ramosa*, −;

Hydrangeoideae, *Hydrangea paniculata*, + +; *H. petiolaris*, + +; *Kirengeshoma palmata*, −; *Philadelphus laxus*, + +; Escallonioideae‖‖, *Escallonia iveyana*, ×; *E. montevidensis*, + +; *E. punctata*, ×; *E. virgata*, ×;

Ribesioideae, *Ribes grossularia*, + +; *R. nigrum*, + +; *R. sanguineum*, + +; *R. speciosum*, (+);

Baueroideae, *Bauera rubioides*, +;

Scrophulariaceae, *Paulownia tomentosa*††, ×; *Veronica acuminata*, −; Simarubaceae, *Ailanthus altissima*, +; *Cneorum tricoccum*, −; *Picrasma quassioides*, −; Staphyleaceae, *Staphylea trifolia*, (+); Sterculiaceae, *Fremontia californica*, −; *Hermannia candicans*, + +; Tamaricaceae, *Tamarix anglica*, (+); *T. tetrandra*, −; Theaceae, *Camellia japonica*, + +; *Stewartia pseudocamellia*, + +; *Thea sinensis*, +; Thymelaeaceae, *Daphne gnidium*, (+); *D. laureola*, −; *D. odora* 'marginata', −; *Pimelia ferruginea*, (+); Tiliaceae, *Tilia europaea*, + +; Tropaeolaceae, *Tropaeolum majus*, −; Ulmaceae, *Ulmus campestris*, + +; Umbelliferae, *Foeniculum capillaceum*, −; *Sium sisarum*, −; Urticaceae, *Boehmeria nivea*, ±; *Parietaria diffusa* (=*officinalis*), + +; *Urtica dioica*, −; Valerianaceae, *Centranthus rubra*, −; *Valeriana sambucifolia*, −; Verbenaceae, *Callicarpa japonica*, −; *Caryopteris tangutica*††, ×; *Clerodendron fargesii*, −; *Diostea juncea*, −; *Petraea volubilis*, −; *Tectona grandis*¶¶, ×; Violaceae, *Hymenanthera crassifolia*, −; *Viola rugulosa*, −; Vitaceae, *Parthenocissus quinquefolia*, + +; Zygophyllaceae, *Peganum harmala*, −; *Zygophyllum fabago*, −.

† Interference due to formation of blue-purple coloration.
‡ Interference due to formation of dark brown coloration, with heavy ppt.
§ Interference due to formation of deep blue-purple coloration.
‖ Young leaves of several species gave positive or indeterminate anthocyanidin reactions, not given by mature specimens, and not confirmed by vanillin reaction.
¶ Interference due to formation of deep brown coloration (cf. Trim & Hill, 1952).
** Contain considerable quantities of catechin-like compounds.
†† Interference due to formation of purplish brown or brown coloration.
‡‡ Interference due to formation of deep steely blue coloration.
§§ Tissue becoming deep reddish brown; interference in vanillin reaction due to intense blue coloration.
‖‖ Interference due to formation of deep blue-black coloration.
¶¶ Interference due to formation of brown-scarlet coloration.
*** Leaves permanently pigmented. Peduncles gave positive reaction with both tests.

Table 2. *Anthocyanidin reaction in leaves of other groups of plants*

See Table 1 for explanation of symbols.

Monocotyledons

Araceae, *Philodendron racemosum*, +; *Zantedeschia elliottiana*, (+); Commelinaceae, *Tradescantia reflexa*, −; Cyperaceae, *Carex pendula*, +; Dioscoreaceae, *Dioscorea opposita*, ++; *Tamus communis*, (+); Gramineae, *Arundinaria japonica*, −; *Saccharum officinarum*†, −; *Sorghum halapense*, −; *S. sachanense*, −; Hydrocharitaceae, *Limnobium stoloniferum*, (+); Iridaceae, *Iris foetidissima*, −; *I. germanica* (cultivar), −; *I. pseudacorus*, ++; Lemnaceae, *Lemna minor*, (+); Liliaceae, *Smilax rotundifolia*, ++; Palmae, *Washingtonia gracilis*, ++; Typhaceae, *Typha latifolia*‡, −; Zingiberaceae, *Elettaria cardamomum*, ++; *Globba winitii*, ±; *Hedychium coccineum*, +; *H. spicatum*, +.

Gymnosperms

Cycadaceae, *Dioon edule*, (+); *Macrozamia riedleri*, ++; *Stangeria eriopus*, −; Ginkgoaceae, *Ginkgo biloba*, ++; Gnetaceae, *Ephedra americana*, +; *Gnetum gnemon*, −; Pinaceae, *Araucaria bidwillii*, +++; *Cedrus deodara*, ++; *Cunninghamia sinensis*, ++; *Metasequoia glyptostroboides*, ++; *Sequoiadendron giganteum*, ++; Taxaceae, *Podocarpus falcatus*, +++; *Taxus baccata*, +++.

Pteridophyta

Equisetaceae, *Equisetum arvense*, −; *E. telmateia*, +; *E. variegatum*, −; Isoetaceae, *Isoetes lacustris*§, ×; Lycopodiaceae, *Lycopodium selago*, −; Marattiaceae, *Angiopteris evecta*, +; *Marattia fraxina*, +; Polypodiaceae, *Dryopteris elongata*, ++; *Pellaea rotundifolia*, ++; *Pteridium aquilinum*, ++; Psilotaceae, *Psilotum triquetrum*, −; Salviniaceae, *Azolla filiculoides*, ++; *Salvinia auriculata*, ++; Schizaeaceae, *Lygodium circinatum*, +; Selaginellaceae, *Selaginella apus*, −; *S. caulescens*, −; *S. kraussiana*, −.

Bryophyta

Dicranum scoparium, −; *Leucobryum glaucum*, −; *Mnium hornum*, −.

† Positive anthocyanidin and vanillin reactions given by roots.
‡ Strongly positive anthocyanidin and vanillin reactions given by roots.
§ Interference due to formation of deep blue coloration.

Discussion of Table 2

Groups other than the dicotyledons have so far been only scantily covered.

In monocotyledons a distinction between woody and herbaceous species is no longer observed; positive and negative reactions are more or less equally divided among the herbaceous forms. In several instances (*Typha*, *Saccharum*) in which the leaves are negative for leuco-anthocyanin, the root-stock reacts positively. All parts of *Iris pseudacorus* except the petals give an intense reaction; neither leaves nor rhizomes of *I. germanica* and *foetidissima*, however, give any reaction. The strong reactions of the fleshy *Dioscorea*, and of the Zingiberaceae are especially remarkable. *Smilax rotundifolia*, although climbing in habit, is essentially woody.

Among the gymnosperms the reaction is general. Its occurrence in *Ginkgo* is worthy of remark, as this is regarded as a form of great antiquity.

In Pteridophyta, the reaction is general among the ferns examined, is present in Equisetaceae, and although any anthocyanidin formed is obscured by a strong darkening of the tissues when heated with hydrochloric acid, judging from the positive vanillin reaction, is also positive in *Isoetes*.

DISCUSSION

That the presence of leuco-anthocyanins in vascular plants is a primitive character is strongly indicated by (1) the generality of their presence in the lower and more primitive forms of these plants—for example, the Equisetaceae, Polypodiaceae, Cycadaceae, *Ginkgo*, and *Metasequoia*—and (2) their absence from those families which, whether woody or herbaceous, are recognized as advanced in their various lines of development. It would seem that the production of leuco-anthocyanins is part of a primitive metabolic pattern associated with, but not essential to, a tree-like or woody habit of growth.

Relationship to lignin

Consideration of a possible role for leuco-anthocyanins in the process of lignification as such is hampered by our present limited knowledge of the chemistry of both leuco-anthocyanins and lignin. As regards the first, there is reason to suppose (cf. Part 1) that the molecule is of the flavonoid C_6–C_3–C_6 pattern substituted with OH in positions C-3 and C-4 or C-3, C-4 and C-5 of one benzene nucleus (see Formulae in Part 1); and as regards lignin, that it is largely composed of C_6–C_3 fragments substituted at C-4 with OH, either alone or with one or both of positions 3 and 5 substituted with OCH_3. It is, in fact, of some importance to have established, as has been done in Part 1, that the leuco-anthocyanins are narrowly restricted, in leaves at least, to unmethylated types. Methylation of the hydroxyl groups at C-3 and C-5 would seem to be an essential feature distinguishing the lignin nucleus from the leuco-anthocyanin (and also the catechin) molecule.

Creighton, Gibbs & Hibbert (1944) and Towers & Gibbs (1953) have made a start on a systematic

survey of the distribution of guaiacyl (4-OH, 3-OCH_3) and syringyl (4-OH, 3:5-di-OCH_3) residues in lignins from a wide range of plants. For the most part, syringyl residues are absent from coniferous lignins (present only in *Podocarpus*) but present, together with *p*-hydroxyphenyl residues, in monocotyledonous lignins and present also in dicotyledonous lignins. Reference to Table 3 of Part 1 will show immediately what little resemblance there is between the systematic distribution of 3:4- and 3:4:5-substituted types in the leuco-anthocyanins and that in the lignins. There is, in fact, as little sense to be made of the distribution of leuco-anthocyanin types in leaves as there is of the different anthocyanin types in flowers (cf. Lawrence, Price, Robinson & Robinson, 1939). The only really outstanding feature of the distribution of leuco-anthocyanin types is, in fact, the absence of leuco-delphinidin from all species of Rosaceae. Otherwise cyanidin and delphinidin types appear to occur indiscriminately within a family, often occurring together in species. It may well be that all these plants possess the capacity, i.e. the enzymic equipment necessary to produce both types, but that in any particular instance one may be formed preponderantly, often to the complete exclusion of the other.

It is important, too, to note that neither type is conspicuously the more 'primitive'. Some Pteridophyta, including *Equisetum*, have leuco-cyanidin alone (or mainly) while others have both types; *Ginkgo* and *Sequoiadendron* have leuco-delphinidin, *Metasequoia*, leuco-cyanidin, other Coniferae both. There is in this context no support for the view (Lawrence *et al.* 1939) that cyanidin is, of the three main anthocyanidin types, the primary (and primitive) product of metabolism; but it must also be borne in mind that there is no evidence that any connexion exists between the metabolism of leuco-anthocyanins and anthocyanins.

The presence of leuco-anthocyanins is evidently not essential to the process of lignification, nor is it essential to the development of a woody habit of growth, since lignification in some degree occurs in all vascular plants at and above the level of Pteridophyta, and many perfectly good trees do not possess leuco-anthocyanins either in the foliage or in the wood itself. Whatever therefore might be the function of leuco-anthocyanins with respect to the development of woody tissue, that function can be assumed by other molecules. It is possible for instance that, in some cases, the coumarins might assume this function. It is probably not merely a coincidence that the recorded occurrence of the coumarins (cf. Bergmann & Gierth, 1932) is virtually confined to those families of plants in the leaves of which leuco-anthocyanins do not occur. If this were so, it would indicate one way in which a primitive metabolic pattern, of which the leuco-anthocyanins form a feature, might be modified in advanced forms without loss of the ability to form wood, but with, perhaps, metabolic economy in such circumstances as the herbaceous condition where suppression of wood formation is a concomitant of their habit of growth.

Relationship to tannin

The relationship of leuco-anthocyanin to 'tannin' in plants is more easily discerned. Amongst naturally occurring substances, the vanillin reaction, one of the reactions used for the histochemical detection of tannins, is given, so far as is known, only by catechins and leuco-anthocyanins. These substances also react in a manner characteristic of tannins with other reagents used for the histochemical detection of tannins, such as ferric salts. It is not surprising, in view of the widespread distribution of the leuco-anthocyanins revealed by the present survey, to find a considerable degree of congruence between their distribution and that recorded for 'tannins' in the botanical literature. The extent of this congruence can be judged from the fact that, out of twenty-five of the families of dicotyledons noted by Metcalfe & Chalk (1950) as regularly tanniniferous, only two (Tamaricaceae and Crassulaceae) have not been found to contain leuco-anthocyanins, and of these, the Crassulaceae contain strongly vanillin-positive, catechin-like substances. The leuco-anthocyanins have, in fact, many properties of tannins besides those mentioned above. They are, for instance, precipitated by hide powder and are astringent in taste (Bate-Smith & Swain, 1953). It is difficult to avoid the conclusion that they are, in fact, the commonest and most typical representatives of the class of substances rather indefinitely described in the botanical literature as 'tannins'. The tannins of commerce are much more definitely characterized under their specific commercial designations, and by their specific property of tanning leather. It is not known to what extent, if any, they owe their tanning properties to leuco-anthocyanins.

Taxonomic implications

Although there would seem to be useful implications for the taxonomist in the results of this survey, it is not intended to attempt to press these to any firm conclusions because, although a wide area has been covered, the number of specimens studied is still so very small. In dicotyledons, confinement of leuco-anthocyanins to Hutchinson's Lignosae (with the exception of a small group of families in Herbaceae) appears to us to provide some validation of the system put forward by Hutchinson (1946) mainly on morphological grounds; and to encourage, perhaps, reconsideration of the placing of

some of the few families containing leuco-anthocyanins which are at present included by him in the Herbaceae.

SUMMARY

1. The presence or absence of leuco-anthocyanins in the leaves of vascular plants is related to their systematic position. They are generally present in ferns, but have not been found in Selaginellaceae, Psilotaceae, or Lycopodiaceae, nor in mosses. They are also generally present in gymnosperms, but are absent from *Gnetum*.* Their occurrence in monocotyledons is common but scattered.

2. In the leaves of dicotyledonous plants, the presence or absence of leuco-anthocyanins appears to be connected with a woody habit in the plant, or in the forms closely related to it. Hutchinson's classification of the families of dicotyledons into a predominantly woody group (Lignosae) and a predominantly herbaceous group (Herbaceae) provides a fairly accurate division into those containing members which have, and those whose members do not have, leuco-anthocyanins in their leaves. There are, however, many members of the Lignosae the leaves of which do not contain leuco-anthocyanins; these are usually either herbaceous in habit, or belong to families containing herbaceous members. A few families of Herbaceae, viz. Saxifragaceae (Hutchinson), Polygonaceae, Oxalidaceae, Limnanthaceae, Balsaminaceae, Aizoaceae and Plumbaginaceae, contain leuco-anthocyanins. In Crassulaceae substances reacting with vanillin occur, but these are not leuco-anthocyanins.

3. The distribution of leuco-anthocyanins in the leaves of Papilionatae has been especially studied. They occur in plants of woody habit in Sophoreae, Dalbergieae, Phaseoleae and Galegeae, and in the herbaceous Hedysareae, but are absent from Podalyrieae, Trifolieae, Loteae and Vicieae. This distribution follows closely the division of Papilionatae by Dormer (1946) on grounds of vegetative morphology.

4. The systematic distribution of leuco-anthocyanins closely follows the recorded incidence of tannins in the botanical literature. They have, in fact, the properties of tannins and are probably the substances most commonly responsible for the reactions in plant tissues attributed to tannins.

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* See also footnote p. 127.

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On the Occurrence of Adrenaline and Noradrenaline in Blood

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Results obtained with a fluorimetric method of estimation (Weil-Malherbe & Bone, 1952, 1953) indicate a concentration of about 2–3 μg. adrenaline and 5–7 μg. noradrenaline/l. human venous plasma under basal conditions. Although these figures are lower than many proposed by previous investigators (see review by Pekkarinen, 1948), even lower levels are postulated by some physiologists. Evidence in support of the specificity of the method has been submitted in our earlier publications (Weil-Malherbe & Bone, 1952, 1953). This paper contains a more detailed report of experiments in which the occurrence and concentration of adrenaline and noradrenaline in blood were studied by paper-chromatographic methods.

A comparison of the R_F values of the fluorogenic substances present in blood with those of adrenaline and noradrenaline provided qualitative evidence

for their identity. Since adrenaline and noradrenaline may be differentiated by the fluorescence ratios obtained with different light filters, a second qualitative criterion was applied by measuring these ratios in the fractions separated by paper chromatography. Particular emphasis was laid on the quantitative aspect of the experiments by drawing up a balance sheet between the amounts determined by the direct fluorimetric method and those recovered from the paper chromatograms and by comparing these recoveries with those of added adrenaline and noradrenaline.

It has been suggested (Lehmann & Kinzius, 1951; Annersten, Grönwall & Köiw, 1949) that plasma contains a biologically inactive, protein-bound form of adrenaline (called 'adrenalinogen' by Lehmann & Kinzius). In view of the fact that precipitation of proteins involves considerable losses of added adrenaline (Gaddum & Schild, 1934; D'Silva, 1937; Lehmann & Michaelis, 1942) an association between adrenaline and plasma proteins is a reasonable assumption. If this association is loose, for instance due to coulombic or van der Waals forces, the complex would be expected to dissociate when the solution containing it is passed through an alumina column or extracted with butanol. Thus it is known that plasma thyroxine is extracted by butanol, although it is non-dialysable and quantitatively adheres to protein precipitates (Taurog & Chaikoff, 1948). However, both procedures failed to liberate the amounts of adrenaline claimed to occur in non-deproteinized plasma by Lehmann & Michaelis (1942, 1949) and by Annersten *et al.* (1949). Since there still remained the possibility of an undissociable link between adrenaline and plasma proteins, such as a peptide bond, it was decided to estimate adrenaline and noradrenaline in partly hydrolysed plasma.

The final problem investigated was the arteriovenous difference of adrenaline and noradrenaline under basal conditions. The rate of adrenaline utilization can be approximately calculated from these figures. As will be shown, there is a good correlation with the known rate of adrenaline discharge from the adrenal gland.

EXPERIMENTAL

Methods

Collection and preparation of blood fractions. Human blood was withdrawn by mixing 3 parts in the syringe with 1 part fluoride–thiosulphate solution (Weil-Malherbe & Bone, 1952). Ox blood was obtained from the slaughter-house; a few minutes after the death of the animal the blood (3 parts) was run from the severed carotid artery into a bottle containing 1 part of fluoride–thiosulphate solution. Plasma was separated by centrifuging. Red cells were washed 3 times with isotonic NaCl and finally suspended in isotonic NaCl made up to the original blood volume. Fluoride–thiosulphate solution was added (1 vol. to 3 of red cell suspension) and cytolysis was induced, in some instances by the addition of cetyltrimethylammonium bromide (cetrimide) solution as previously described (Weil-Malherbe & Bone, 1953), or, more frequently, by freezing at $-40°$ in an ethanol–dry ice bath (Lovelock, 1953). The cytolysed solution was finally clarified by centrifuging.

The packed-cell volume of the mixture of blood and fluoride–thiosulphate solution was determined by centrifuging in haematocrit tubes for 45 min. at approx. 2000 *g*.

Extraction. Samples of plasma and erythrocyte lysates were extracted with 2 vol. *n*-butanol by vigorous mechanical shaking in stoppered centrifuge tubes for 10 min. The emulsion was broken by centrifuging and the clear supernatant transferred to a separating funnel. The residue, including a creamy intermediate zone, was extracted twice more with butanol (2 vol.). The pooled butanol extracts (6 vol.) were extracted 3 times with 0·05 N-HCl (2 vol./extraction). The combined HCl extracts were concentrated *in vacuo* to about a third of their volume and the pH was adjusted to 3 by the gradual addition, with constant stirring, of Dowex 2 (OH form, 20 to 40-mesh; Microchemical Specialities Co., Berkeley, California). After the resin had been filtered off and repeatedly washed with water, the filtrate and washings were further concentrated *in vacuo* until the volume was reduced to about one-fifth of that of the blood sample used.

To the concentrated extract (50 ml.) were added 0·2 M phosphate buffer pH 6·5 (5 ml.) and enough N-NaOH to raise the pH to 6·5; 3% (w/v) $Na_2S_2O_3$ (5 ml.) was also added. Any precipitate formed (calcium phosphates?) was removed by centrifuging. The solution was adjusted to pH 8·4 by careful addition of 0·2 N-NaOH and passed through a column of acid-washed aluminium oxide. The preparation of this column and the procedure of adsorption and elution were according to Weil-Malherbe & Bone (1952).

Comment on extraction procedure. The use of butanol as solvent was suggested by the method of extracting thyroxine from plasma (Taurog & Chaikoff, 1948). Adrenaline, like thyroxine, is carried down by protein precipitates, a fact which precludes the use of trichloroacetic acid and similar agents.

By partially neutralizing the HCl extract with Dowex 2 the introduction of salt is avoided, but this step is safe only if certain precautions are observed. If the HCl extract is treated with resin while it is still saturated with butanol, adrenaline is lost through adsorption by the resin. After the extract has been concentrated to 0·3–0·5 vol., the butanol has been quantitatively distilled off. If, on the other hand, concentration of the solution is carried too far, adrenaline may again be adsorbed by the resin; this may occur when the HCl concentration exceeds 0·2 N. Rapid mechanical stirring is necessary to ensure a thorough mixing while the resin is slowly added, thus avoiding local zones of alkalinity. The amount of resin required to neutralize a given quantity of acid is fairly constant and should be determined in advance.

Several other anion-exchange resins were found unsuitable since they released fluorescent material.

Paper chromatography. The eluate from the column of alumina was evaporated to dryness *in vacuo*, the process being finished in a small test tube, 75 × 12 mm. The residue was extracted 3 times with 0·5 ml. acid acetone (1 ml. conc. HCl in 100 ml. acetone) and the extract transferred to the starting line of the paper strip in 5 μl. portions under a jet of N_2 to accelerate drying.

Chromatography was carried out by the ascending technique in Pyrex jars, 22 × 9 in., fitted with a ground flange and a tubulated desiccator lid. The jars stood in a thermostatically controlled cabinet at 23°. Each jar held three separate paper strips, usually a reference strip, a blank strip and the strip carrying the blood extract. The strips were suspended from a glass-rod triangle by means of a bent-over flap and were secured by a length of glass rod threaded through slots. A glass rod was also threaded through the paper below the line of application. The paper strips measured 48 × 8 cm.; the line of application was at a distance of 6 cm. from the lower edge.

Whatman no. 1 paper was used for most experiments. The strips, in batches of about 50, were bathed in 0·5 N-HCl (three changes) for 24 hr. and washed in several changes of distilled water. They were then immersed in a solution of 0·1 % ethylenediaminetetraacetate for 24 hr., washed and air-dried. In some experiments with butanol–HCl mixture as solvent the paper used was Grycksbo OB.

A mixture of phenol, freshly distilled over aluminium (Draper & Pollard, 1949), and glass-distilled water (3:1, w/v) was used as solvent in most experiments. A dish containing 5 N-HCl, saturated with phenol, was placed in the bottom of the jar. In some experiments butanol saturated with N-HCl was used as solvent (Hamberg & Euler, 1950). At the end of the run (after 21–24 hr.) the paper strips were twice immersed in benzene (purified by extraction with conc. H_2SO_4 and redistilled) and hung up to dry for 0·5 hr. The reference strip was sprayed with the potassium ferricyanide reagent of James & Kilbey (1950), while the strip containing the blood extract was cut into a series of eight to nine transverse sections: two of these corresponded to the zones occupied by the adrenaline and noradrenaline spots on the reference strip, one corresponded to the clear interval between the spots and the remainder accounted for the paper between the line of application and the noradrenaline spot and that between the adrenaline spot and a line situated about 2–3 cm. below the solvent front. Each section was placed in a test tube containing 10 ml. 0·01 N-HCl and extracted for 12–24 hr. at 3°. Each HCl extract was shaken with three lots of 10 ml. freshly distilled, peroxide-free ether to remove traces of phenol. The extracts were freed from ether by brief evacuation and carried through the condensation with ethylenediamine by the procedure described (Weil-Malherbe & Bone, 1952).

Comment on the chromatographic procedure. The method described is essentially that of Goldenberg, Faber, Alston & Chargaff (1949). Solvent systems consisting of mixtures of butanol with water and various acids (formic, acetic, oxalic and HCl) did not lead to clear-cut separations of the adrenaline and noradrenaline spots in the ascending method. Attempts to use the descending method were abandoned, since the rate of flow was liable to differ in the separate paper strips, a complication not encountered with the ascending technique.

Various modifications of the phenol method were tested. Phenol containing 15 % (v/w) 0·1 N-HCl (Vogt, 1952) slightly decreased the R_F value of both adrenaline and noradrenaline. Filling the jar with CO_2 (Vogt, 1952) improved recoveries a little and this procedure was adopted in later experiments. A slight improvement of recovery was also found when the papers were sprayed with ascorbic acid according to Crawford & Outschoorn (1951) (Expt. 4, Table 5), but this was offset by a wider spread of the spots. The treatment was therefore omitted. None of the modifications reduced the fluorescence of the blank.

A factor of importance for obtaining low blank readings was the purity of the benzene and ether used for the removal of phenol. The use of ether contaminated by peroxides resulted in highly fluorescent blanks, presumably owing to the formation of oxidation products of phenol.

Fluorimetric estimation. This was carried out as described in previous papers, in the earlier experiments by the original method (Weil-Malherbe & Bone, 1952), later by the differential method (Weil-Malherbe & Bone, 1953).

In the differential method, fluorimeter readings are taken first with a yellow secondary filter (Chance OY 4, maximum transmission 550 mμ. and upwards) and next with a blue-green filter (Ilford Bright Spectrum Filter 623, max. transmission approx. 490 mμ.). While the fluorescence of the noradrenaline derivative is of similar intensity at both wavelengths that of the adrenaline derivative is about 4 times stronger in the yellow than in the green region. The results are calculated with the aid of simultaneous equations which, on solution, yield the following formulae:

$$\text{Noradrenaline } (N) = mn(b - y)/(m - n), \quad (1)$$

$$\text{Adrenaline} = y - N/m = b - N/n, \quad (2)$$

where m = ratio of adrenaline/noradrenaline fluorescence with yellow filter; n = ratio of adrenaline/noradrenaline fluorescence with blue-green filter; y = amount of adrenaline corresponding to fluorescence measured with yellow filter; b = amount of adrenaline corresponding to fluorescence measured with blue-green filter. Unfortunately, equation (4) of the paper of Weil-Malherbe & Bone (1953), corresponding to equation (2) above, was erroneously printed with brackets and this opportunity is taken to correct the error.

Other amendments of previous directions are as follows:

(1) Ethylenediamine dihydrochloride is obtained in better yield by dissolving 50 ml. freshly distilled ethylenediamine in 200 ml. ethanol and mixing this solution with 1500 ml. ethanolic HCl containing 150 ml. conc. HCl. After cooling the crystals are filtered off, washed with ethanol and dried.

(2) If the *iso*butanol extract is exposed to atmospheric CO_2 for any length of time, a turbidity is liable to appear owing to formation of ethylenediamine carbonate.

RESULTS

Recovery of adrenaline and noradrenaline. Recoveries were studied separately in the two phases of the analysis, extraction and paper chromatography. The efficiency of the extraction procedure was examined by analysing a sample of the eluate from the alumina column. The results (Table 1) show that adrenaline added to plasma or serum is recovered in 75–85 % yield. The recovery of the unknown fluorogenic material from plasma is of the same order (Table 1, bottom line). Losses, however, tend to become larger when the scale of the process is increased (cf. Table 6). A slightly lower recovery is also found when adrenaline is added to red cell extracts. This may be due to the fact that haemoglobin when treated with butanol turns into a sticky mass which it is impossible to disperse.

Table 1. *Recovery of adrenaline by extraction procedure*

In experiments where adrenaline was added the results were corrected by subtracting 'pre-formed' adrenaline determined in a control sample. In experiments without addition (last line of table) the recovery figures are based on a comparison between an analysis of a plasma sample before extraction and an analysis of the purified extract.

No. of expts.	Blood fraction	Volume extracted (ml.)	Adrenaline added (μg.)	Mean recovery (%)	S.E.M.
3	Horse serum	20	10	74	—
1	Horse serum	20	5	85	—
7	Red cells (human)	20–40	1	66·3	2·3
2	Plasma (human)	20	0·2	82·9	—
10	Plasma (human)	70–115	0	80·2	4·0

Table 2. *Recovery of adrenaline and noradrenaline by paper chromatography*

In Expt. 1 the amines were applied directly to the paper. In Expt. 2 they were contained in 0·2 ml. water and in Expts. 3 and 4 in 15 ml. 0·067 M acetic acid. The solutions were evaporated to dryness and the residue was extracted with acid acetone. The fluorimetric assay of the eluates from the paper sections was performed with the yellow filter only.

	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Adrenaline added (μmg.)	100	200	80	160
Noradrenaline added (μmg.)	500	1000	300	600
Total added in terms of adrenaline fluorescence (μmg.)	214	427	148	296
Recovered: (μmg. in terms of adrenaline fluorescence)				
Residue in tube	—	0	13	15
In noradrenaline section	106	164	43	94
In adrenaline section	98	177	68	103
Total recovered	204	341	124	212
Recovery (%)	96	80	84	71·5

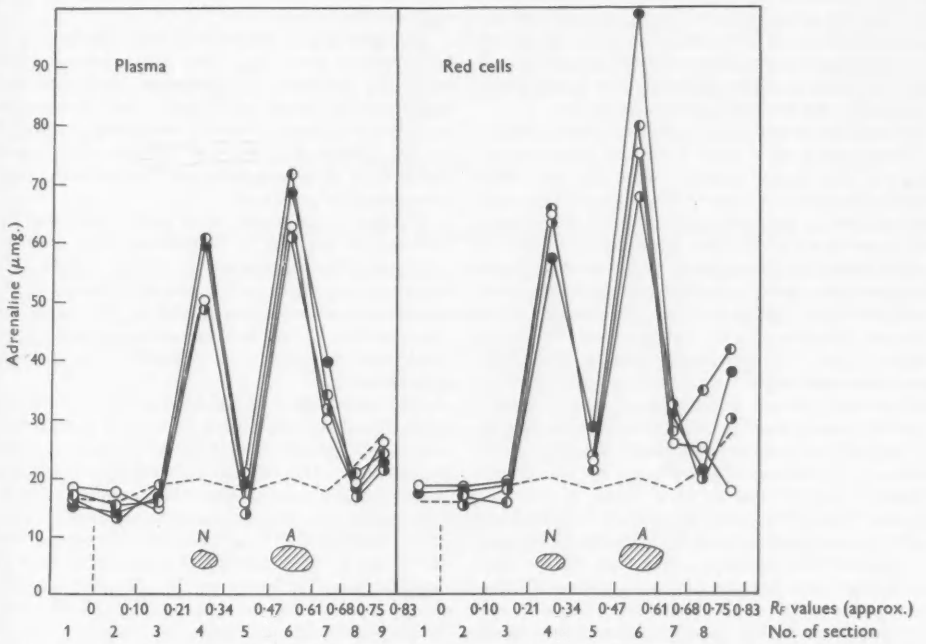

Fig. 1. Paper chromatography of extracts of human plasma and red blood cells. Fluorimetric readings (in terms of μmg. adrenaline) obtained in four experiments are plotted against the approximate R_F values of the cuts. Shaded areas: position of noradrenaline (*N*) and adrenaline (*A*) spots on guide strip.

The recovery of adrenaline and noradrenaline by the paper-chromatographic method was studied in other experiments (Table 2). These experiments were done before the differential method (Weil-Malherbe & Bone, 1953) was available and the estimations are based on fluorimeter readings obtained with a yellow filter. Under these conditions adrenaline gives rise to a fluorescence intensity which is about 4·4 times greater than that given by noradrenaline. This factor was used to calculate the balance of recovery. All readings were corrected by subtracting the appropriate blank. It appears from Table 2 that losses are small during the paper-chromatographic procedure itself, but that some losses are incurred during the preceding stage of evaporation and transfer (see also Table 5).

Analysis of blood extracts. In a first series of experiments, carried out before the development of the differential method, plasma and red cell fractions of ten human blood samples measuring 120–150 ml. were analysed. The qualitative aspect of the results is apparent from Fig. 1, where the fluorescence intensities produced by the eluates (in terms of μmg. adrenaline) are plotted against the approximate R_F value of the cuts (the position of the cuts is not accurately drawn to scale since it varied somewhat from experiment to experiment). The shaded areas indicate the appearance of the noradrenaline and adrenaline spots on the guide strip. Without exception the analysis of both plasma and corpuscle extracts produced two peaks which coincided with the position of these spots.

Some fluorescence also appeared in the eluates of a blank paper strip (Fig. 1, broken line). Its intensity was fairly constant and did not vary appreciably with the size of the paper section, with its position up to an R_F value of about 0·7, or from one experiment to another. Between R_F 0·7 and the solvent front the fluorescence of the blank tended to increase. This effect coincided with the appearance, in these paper sections, of the 'pink front', which formed irregular-shaped streaks near the upper edge of the chromatogram. Despite many precautions, such as the use of acid-washed, ethylenediaminetetraacetate-treated paper and of freshly distilled phenol and the displacement of air by CO_2, the pink front could not be entirely eliminated. The amount of fluorescence produced by the eluates from the upper regions of a blank paper strip showed little uniformity in different experiments and contaminated sections were therefore discarded.

Eluates from sections other than those corresponding to the position of the adrenaline and noradrenaline spots on the guide strip did not yield a fluorescence higher than the general level of the blank. This is particularly significant in the case of the section intermediate between adrenaline and noradrenaline. The only exception was the section following adrenaline (no. 7 in Fig. 1), which often showed a small amount of fluorescence above the blank level. Since the spots tend to spread with increasing R_F and the section was contiguous with the adrenaline section it was assumed that the fluorescence was produced by the same substance as that in the adjoining fraction. Any excess fluorescence found in this section was therefore calculated as adrenaline. It was later found by the differential technique that this procedure was justified.

From a qualitative point of view, it may thus be stated that the fluorogenic matter of the extracts is entirely concentrated in positions corresponding to those of adrenaline and noradrenaline, at any rate up to an R_F value of about 0·8. A quantitative balance sheet is shown in Tables 3 and 4. The amount of fluorescence recovered in the adrenaline and noradrenaline positions of the paper chromatogram and expressed in terms of μmg. adrenaline has been compared with the amount of fluorogenic matter (also in terms of μmg. adrenaline) present in the purified extract before evaporation. Included in the balance is the residue left in the test tube after extraction with acid acetone. It was dissolved in water, treated with ethylenediamine and analysed fluorimetrically. It accounted for about 10 % of the recovered matter.

Extracts of both plasma and red cells gave mean recoveries of over 70 %. The figures compare well with the recovery of authentic adrenaline and noradrenaline shown in Table 2. There is therefore no reason to assume that the substances estimated in the fluorimetric method include components other than those migrating to the adrenaline and noradrenaline positions.

Further experiments were performed after the differential method of estimation had been developed (Weil-Malherbe & Bone, 1953). On applying the formula for the calculation of the results, the material extracted from the noradrenaline position should analyse as noradrenaline and that isolated from the adrenaline position as adrenaline.

The fluorescence of the blank when determined with the blue-green filter was even higher than when determined with the yellow filter; it corresponded with the fluorescence given by 60–80 μmg. adrenaline, increasing near the upper edge of the chromatogram. Similar blank values were obtained with butanol–N-HCl as solvent. Readings were corrected by subtracting the blank value, and the calculations were based on the corrected readings. These corrections were necessarily fraught with some inaccuracy as shown, for instance, by the occasional appearance of a negative result or by the appearance of positive figures at positions separated from the main fraction by a gap. Such results were

Table 3. *Paper chromatography of adrenaline and noradrenaline in human plasma extracts*

Fluorimetric assay with yellow filter. Results expressed in terms of μmg. adrenaline. The plasma extracts of Expts. 6 and 10 were lost.

Expt. no.	Plasma extracted (ml.)	Residue	Zone of application	Noradrenaline zone	Adrenaline zone	Total recovered	Content of extract (before evaporation)	Recovery (%)
1	70	15	0	59	105	179	209	85·6
2	74	8	2	55	76	141	158	89·0
3	95	14	0	50	69	133	239	55·7
4	82	13	4	45	82	144	214	67·3
5	84	6	0	45	73	124	182	68·3
7	76	15	0	45	66	126	161	78·3
8	83	13	0	41	63	117	186	63·0
9	80	20	0	45	60	125	177	70·8
			Mean % recovery (with S.E.)					72·2±4·0

Table 4. *Paper chromatography of adrenaline and noradrenaline in human erythrocyte extracts*

Fluorimetric assay with yellow filter. Results expressed in terms of μmg. adrenaline.

Expt. no.	Volume extracted (ml.)	Residue	Zone of application	Noradrenaline zone	Adrenaline zone	Total recovered	Content of extract (before evaporation)	Recovery (%)
1	120	12	0	73	86	171	218	78·5
2	100	13	6	50	72	141	179	79·0
3	134	19	0	47	51	117	242	48·5
4	90	16	0	45	65	126	238	53·0
5	106	15	0	49	80	144	185	78·0
6	104	23	0	45	66	134	199	67·4
7	104	16	0	52	62	130	180	72·3
8	104	19	0	54	72	145	201	72·0
9	104	25	0	51	63	139	181	77·0
10	115	17	0	49	91	157	187	84·0
			Mean % recovery (with S.E.)					71·0±3·7

Table 5. *Recovery of adrenaline and noradrenaline by paper chromatography*

Fluorimetric assay by differential method. Adrenaline and noradrenaline solutions were directly applied to the paper in Expts. 1–4. In Expts. 5 and 6 they were added to 15 ml. 0·067 M acetic acid and evaporated before application to paper. In Expt. 4 the paper was sprayed with ascorbic acid solution before use. Bracketed values were omitted from calculations.

Expt. no.	Added	Recovered in: Residue	Noradrenaline zone	Adrenaline zone	Total	Recovery (%)
			Adrenaline (μmg.)			
1	1000	—	0	830	830	83
2	100	—	(2)	64	64	64
3	100	—	0	83	83	83
4	100	—	(1)	99·5	99·5	99·5
5	600	20	(−15)	301	321	53·5
6	600	5	(2)	330	335	56
			Noradrenaline (μmg.)			
1	1000	—	688	0	688	68·8
2	100	—	66	(7)	66	66
3	100	—	86	(13)	86	86
4	100	—	90	0	90	90
5	900	56	571	0	627	69·7
6	900	17	509	(12)	526	58·5

Table 6. *Balance sheet of extraction and paper chromatography of adrenaline and noradrenaline in plasma*

Fluorimetric assay by differential method (see text). Bracketed values were considered erroneous and were omitted from balance calculation. All quantities in columns 2–8 are in μmg. unless otherwise stated.

Expt. no.		1		2		3		4	
Species		Bovine		Bovine		Bovine		Human	
Volume extracted (ml.)		1080		1000		800		120	
Corresponding volume of undiluted plasma (ml.)		675		650		491		86	
		Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Estimated before extraction		2770	5660	2520	3810	2960	4400	263	364
Estimated in extract		2400	3520	1270	1990	1900	2350	201	333
Recovery in extract (%)		86·6	62·2	50·5	52·2	64·3	53·5	76·5	90·6
Paper chromatography with phenol									
Amount used		1170	1710	617	974	1770	2250	181	300
Paper sections									
No.	R_F (approx.)								
1	Below 0–0·09	0	0	0	0	0	0	0	0
2	0·09–0·22	0	0	0	0	0	0	0	0
3*	0·22–0·34	(10)	275	(15)	199	(11)	830	(−2)	119
4	0·34–0·48	(−4)	97	(11)	46	(−4)	59	0	0
5†	0·48–0·64	157	(12)	189	0	532	(−52)	81	0
6	0·64–0·74	76	0	10	(7)	9	0	13	0
7	0·74–0·84	0	0	0	0	0	0	0	0
8	0·84–0·93	0	0	0	0	0	0	0	0
Residue after acetone extraction		323	512	76	123	268	326	25	43
Total		556	884	275	368	809	1215	119	162
Recovery (%)		47·6	51·6	45·5	38	45·6	54·0	65·8	54·0
Paper chromatography with butanol–HCl									
Amount used		1170	1710	617	974				
Paper sections									
No.	R_F (approx.)								
1	0·11–0·14	0	0	0	0				
2	0·14–0·17	(4)	52	0	0				
3*	0·17–0·20	26	103	(−12)	108				
4*	0·20–0·23	36	89	10	126				
5†	0·23–0·26	55	40	60	38				
6†	0·26–0·29	49	28	98	12				
7	0·29–0·32	42	0	20	(18)				
8	0·32–0·35	12	(10)	8	(2)				
9	0·35–0·39	0	0	0	0				
10	0·39–0·43	0	0	0	0				
Residue after acetone extraction		261	278	146	290				
Total		481	590	352	574				
Recovery (%)		41·2	34·5	57·0	59·0				

* Sections corresponding to position of noradrenaline spot on guide strip. † Sections corresponding to position of adrenaline spot on guide strip.

considered erroneous and they are shown in Tables 5 and 6 in brackets. It will be noticed, however, that the rejected figures are less than 20 μmg. and that their inclusion would not significantly alter the result.

Recoveries of added adrenaline and noradrenaline were again lower when the solutions were evaporated to dryness before application to the paper (Table 5, Expts. 5 and 6). As was to be expected in recovery experiments, the substance extracted from the noradrenaline position did not contain significant amounts of adrenaline and *vice versa*.

The results of three extractions of bovine plasma and one of human plasma are recorded in Table 6. With the larger quantities of bovine plasma available, more concentrated extracts could be used for paper chromatography, and the relative extent of the corrections imposed by the blank accordingly reduced. Moreover, in two experiments the extract was divided and simultaneously chromatographed with both solvent systems. In the butanol chromatogram the adrenaline and noradrenaline spots on the reference strip were partly overlapping; a length of 10 cm. was cut out of the plasma strip in such a way that the middle zone corresponded to the position of the spots on the reference strip. The paper was then cut into ten equal transverse sections of 1 cm. width. The phenol chromatogram was cut into sections as previously described.

The recoveries in these large-scale experiments were lower than in the analyses of human plasma. There was also a larger proportion of both amines remaining in the undissolved residue after acetone extraction. Nevertheless, the recoveries did not fall far short of, or were equal to, the figures found in model experiments.

The fluorescence ratio obtained with the extracts from the noradrenaline positions was that of the noradrenaline derivative and accordingly the calculation showed adrenaline to be absent from these fractions, within the limits of error. The same is true, *mutatis mutandis*, for the extracts from the adrenaline positions. Separation was incomplete in the butanol chromatogram, but the concentration curves of adrenaline and noradrenaline had different peaks which coincided with the position of the respective spots on the guide strip.

Expt. 4, Table 6, shows that entirely satisfactory results can be obtained when the differential method is applied to extracts from about 80 ml. human plasma.

Adrenaline and noradrenaline in partially hydrolysed plasma. Preliminary experiments showed that 95 % of adrenaline was recovered after refluxing in 2N-HCl for 6 hr., while an increase in the concentration of HCl led to considerably lower recoveries. When plasma was refluxed in 2N-HCl the mixture first became flocculent and turbid, but later cleared and was perfectly homogeneous after 6–8 hr. Though hydrolysis was presumably incomplete, at least a partial liberation of bound adrenaline might have been expected.

The experiments (Table 7) were carried out with bovine plasma. A sample was mixed with an equal volume of 4N-HCl and refluxed for 8 hr. After cooling, the solution was neutralized by the slow addition, with vigorous stirring, of 8N-NaOH. A second sample of equal size was mixed with an equal volume of 4N-NaCl and made up to the same volume as the first sample. Both samples were extracted with butanol and the procedure continued as described. The purified extracts were finally chromatographed on paper with phenol as solvent and the eluates from the paper sections analysed by differential fluorimetry. The agreement of the results obtained with the hydrolysed and unhydrolysed samples is as good as can be expected. These experiments therefore do not support the hypothesis of a protein-bound form of adrenaline existing in plasma.

Several unsuccessful attempts were made to achieve a more thorough hydrolysis by digesting plasma with a cation-exchange resin (Dowex 50) in 0·05N-HCl at 100°, but although adrenaline could be recovered satisfactorily from pure solution, a plasma suspension remained flocculent for up to 6 days.

Arteriovenous differences of adrenaline and noradrenaline in plasma. Arteriovenous differences were determined in six fasting human subjects, all males (Table 8). The adrenaline level was always higher in arterial than in venous plasma by 0·5–1·0 μg./l. The noradrenaline level of the arterial plasma, on the other hand, was found to be higher than in venous plasma in three cases and lower in the

Table 7. *Estimation of adrenaline and noradrenaline in hydrolysed and unhydrolysed bovine plasma*

Fractionation by paper chromatography. Fluorimetric assay by differential method. Amounts given are in μmg.

Expt. no.	Diluted plasma (ml.)	Unhydrolysed		Hydrolysed	
		Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
1	50	35·2	106	38·6	158
2	100	65	194	51	140

Table 8. *Arteriovenous differences in the adrenaline and noradrenaline content of human plasma*

A = arterial blood; V = venous blood; Δ = arteriovenous difference.

Expt. no.	Adrenaline (μg./l. plasma)			Noradrenaline (μg./l. plasma)		
	A	V	Δ	A	V	Δ
1	3·26	2·20	1·06	5·38	6·82	−1·44
2	3·68	2·73	0·95	6·44	5·58	0·86
3	3·76	3·10	0·66	4·40	4·60	−0·20
4	3·80	3·30	0·50	4·40	6·92	−2·52
5	2·49	1·77	0·72	6·32	5·65	0·67
6	2·47	1·87	0·60	6·15	4·98	1·17

other three cases. Although the number of experiments is small, it is felt that the results are probably significant, especially in view of the consistency of the adrenaline results. A negative arteriovenous difference for noradrenaline does not seem improbable if it is remembered that noradrenaline is discharged not only from the adrenal medulla, but also from the sympathetic nerve endings of peripheral tissues.

DISCUSSION

The work described shows that plasma and red blood cells contain two components one of which has the R_F value of adrenaline, the other that of noradrenaline in two solvent systems. Both fractions yield, on treatment with ethylenediamine, fluorescent derivatives whose fluorescence ratios at two different wavelengths agree with those of the adrenaline and noradrenaline derivatives, respectively. Quantitatively these fractions accounted, or almost accounted, for such a proportion of the adrenaline and noradrenaline estimated by the routine procedure as corresponded to the recovery of added amines in model experiments.

Though they do not supply incontrovertible proof, these facts strongly support the supposition that the blood constituents estimated by the fluorimetric method are identical with adrenaline and noradrenaline. Several attempts were made to test the biological activity of the fractions eluted from the paper chromatogram. An experiment in which the rat-uterus method was used has been described in an earlier publication (Weil-Malherbe & Bone, 1952); it was inconclusive owing to the presence of interfering material. The same difficulty arose when a noradrenaline fraction, isolated by paper chromatography from about 80 ml. human plasma, was tested by the rat blood-pressure test; again the extract contained substances which interfered with the action of added noradrenaline. (This experiment was kindly performed by Dr M. Vogt.) Finally, eluates from the adrenaline and noradrenaline zones of a paper chromatogram were tested by Dr R. T. Grant by measuring the constrictor action on the artery of the denervated rabbit's ear (Armin & Grant, 1953). The noradrenaline fraction produced, after a transitory dilator effect, a constriction which was equal to that produced by a standard solution of the expected strength ($1:10^{-11}$). When, however, the adrenaline fraction was tested, a strong and irreversible dilatation of the artery ensued. An extract from the corresponding position of a paper blank had the same effect. The interfering material which was obviously derived from the reagents probably accumulated in the more distal parts of the chromatogram, as does the fluorescent impurity and the 'pink front'. It is intended to continue these experiments with more highly purified fractions.

A method for the estimation of adrenaline and noradrenaline in blood which in sensitivity and specificity is comparable to that used in this laboratory has been described by Lund (1949), but this author was unable to detect measurable amounts of these amines in peripheral venous blood. Lund's method was therefore compared with the ethylenediamine method by analysing samples of the same specimen of plasma. The results were practically identical (Bone, 1953).

The results of Table 8, expressed in μg./l. of whole blood, yield a mean value of 0·50 for the adrenaline arteriovenous difference. Assuming a basal minute volume of 4 l., a mean body weight of 60 kg., and an identical arteriovenous difference in all parts of the vascular system, an adrenaline utilization rate of 0·033 μg./kg./min. is obtained. This figure may be compared with the adrenaline output from a single adrenal gland of the anaesthetized cat, which was found to be 0·015 μg./kg./min. by Dunér (1953) and about 0·02 μg./kg./min. by Kaindl & Euler (1951), or of the anaesthetized dog, which is 0·011–0·027 μg./kg./min. according to Houssay & Rapela (1953). Wada & Kanowoka (1935) reviewed the results of the Japanese school, obtained on dogs without the use of an anaesthetic, and gave as the mean of 377 observations the figure of 0·06 μg./kg./min. for the basal adrenaline discharge from both adrenal glands.

In a first series of blood samples a mean concentration of plasma noradrenaline of about 5·2 μg./l. blood was found (Weil-Malherbe & Bone, 1953). From a large number of analyses since carried out it appears that this figure was probably a little high and that the true mean is approx. 4·5 μg./l. blood.

On the basis of a plasma volume of 55% of blood and expressed in molar units the concentration of adrenaline in plasma is about 0·015 μM, that of noradrenaline about 0·048 μM. It is of interest to compare these figures with the concentration of other low-molecular hormones in plasma, in so far as they have been determined by reliable methods. The concentration of thyroxine may be calculated if it is assumed as a first approximation that all protein-bound iodine is accounted for by thyroxine. From a value of 4–8 μg./100 ml. of protein-bound iodine in plasma (Kydd, Man & Peters, 1950; de Mowbray & Tickner, 1952) the concentration of thyroxine is calculated as 0·08–0·16 μM. Recent estimates of individual adrenocortical steroids in plasma (Morris & Williams, 1953; Bliss, Sandberg, Nelson & Eik-Nes, 1953; Bush & Sandberg, 1953; Bayliss & Steinbeck, 1953) suggest that they occur in concentrations of 0·06–0·30 μM. In order of magnitude the concentration of adrenaline and noradrenaline in plasma is therefore comparable with that of other hormones.

Note added in proof. Recent experiments have shown that 'plasma adrenaline' and 'plasma noradrenaline' are largely associated with the blood platelets and are liberated under the conditions of blood collection described. It has also been confirmed, with various blood fractions and for both adrenaline and noradrenaline, that the method of Lund (1950) gives, in our hands, results identical with those obtained by the ethylenediamine method within the limits of error.

SUMMARY

1. Methods are described for the extraction and fractionation of adrenaline and noradrenaline from plasma and erythrocytes.

2. It has been shown, using two different solvent systems, that the blood constituents estimated by the fluorimetric method of Weil-Malherbe & Bone (1952, 1953) are concentrated on paper chromatograms in positions corresponding to the R_F values of adrenaline and noradrenaline.

3. The fraction migrating with the R_F value of noradrenaline yields a derivative the fluorescence of which has the same spectral properties as the derivative of noradrenaline. The same correlation exists between adrenaline and the fraction having the R_F value of adrenaline.

4. The quantity of the substances estimated as adrenaline and noradrenaline by the routine procedure was compared with the quantity of the substances recovered in the adrenaline and noradrenaline zones of the paper chromatogram. The proportion accounted for was similar to the recovery of added amines in model experiments. It is concluded that the results are compatible with the identity of the blood constituents estimated with adrenaline and noradrenaline.

5. No increase of adrenaline or noradrenaline was found after the partial hydrolysis of plasma by boiling in 2N-HCl. This result does not support the existence of a protein-bound fraction of adrenaline in plasma.

6. Arteriovenous differences were determined in six human subjects. The arterial concentration of adrenaline was always higher than the venous concentration. The noradrenaline concentration was higher in arterial than in venous plasma in three cases, while the reverse was found in the other three cases.

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Reconstruction of the Succinic Oxidase System from Two Preparations, One Deficient in Succinic Dehydrogenase, the Other Deficient in Cytochrome Oxidase

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The best material for the study of the succinic oxidase system, which is bound to the insoluble structural elements of the cell, is the cell-free heart muscle preparation of Keilin & Hartree (1947). It consists probably of fragmented sarcosomal membranes (Cleland & Slater, 1953). Preparations of similar nature can be obtained from a variety of aerobic cells. It is believed that each particle contains all the catalysts necessary for the oxidation of succinate, but to what extent the enzymes present in one particle can react with enzymes of another particle in the course of hydrogen transfer from succinate to oxygen is not known. One way to study this problem of interaction of particles is to mix two preparations, each one inactive in the oxidation of succinate by being deficient in a different component of the enzyme system, thus trying to reconstruct the complete succinic oxidase system. Although such preparations have been obtained in the past (Keilin & Hartree, 1940), the treatments used to destroy some of the enzymes (for instance incubation with 45% aqueous ethanol for 10 min. to destroy cytochrome oxidase) undoubtedly modify the colloidal structure. It is, therefore, not surprising that the attempts made in this direction have so far failed.

While the classical succinic oxidase system, as in heart muscle, is found in wild type *Neurospora crassa*, preparations from the mutant strains designated *C 117* and *poky* are deficient in succinic oxidase and cytochrome oxidase activities, though succinic dehydrogenase is as active as in the wild type (Haskins, Tissières, Mitchell & Mitchell, 1953; Tissières & Mitchell, 1954). On the other hand, a heart muscle or wild-type *Neurospora* preparation deficient in succinic dehydrogenase can easily be obtained by incubation at alkaline pH (Keilin & Hartree, 1940).

In this paper, spectroscopic and manometric experiments on the succinic oxidase system as reconstructed from these two preparations, one deficient in cytochrome oxidase, the other deficient in succinic dehydrogenase, will be described and discussed.

MATERIAL AND METHODS

The origins of the *Neurospora* strains *poky* and *C 117* have been described by Mitchell & Mitchell (1952) and Mitchell, Mitchell & Tissières (1953), respectively. The mould was cultivated in aerated liquid medium (Haskins *et al.* 1953) and the enzyme preparation isolated according to Tissières & Mitchell (1954). Heart muscle preparation was made from horse heart according to the procedure of Keilin & Hartree (1947). Preparations deficient in succinic dehydrogenase were obtained as described by Keilin & Hartree (1940) with the following modifications: it was found necessary to bring the pH to 9·3–9·4 with 0·1 N-NaOH and let the preparation stand for 75 min. at room temperature (18–20°) to destroy succinic dehydrogenase activity almost completely. With wild-type *Neurospora*, the same result was obtained by incubation at pH 9·1–9·2 for 60 min. The preparations were neutralized by addition of 0·2 M-KH_2PO_4. Estimations of cytochrome oxidase, succinic oxidase and succinic dehydrogenase were carried out in Barcroft differential manometers at 35°, in a reaction mixture including the enzyme, of total volume 3 ml., with 0·075 M phosphate buffer, pH 7·0.

Cytochrome oxidase. The manometric flask contained 4×10^{-5} M cytochrome *c* and, in a dangling tube, neutralized ascorbic acid (5%, w/v, 0·3 ml.). The dangling tube was dislodged after temperature equilibration, at zero time.

Succinic oxidase. The dangling tube contained 0·3 ml. 0·4 M succinate; cytochrome *c* was omitted in some experiments.

Succinic dehydrogenase. The flask contained no cytochrome *c*, but 0·001 or 0·002 M methylene blue and 0·003 M cyanide were present, and the dangling tube contained 0·3 ml. 0·4 M succinate.

The cytochrome spectrum was examined by means of a Zeiss microspectroscope mounted on a microscope, as described by Keilin & Hartree (1946). Fat-free dry weight was obtained according to the procedure used by Slater (1949).

RESULTS

Spectroscopic observations

C117 *preparation.* The cytochrome system in *C 117* preparations has been studied in some detail by Tissières & Mitchell (1954). On addition of a small amount of succinate, the absorption bands of cytochromes *b* and *e*, the only components found in

this strain of *Neurospora*, became quite strong. Their intensity was not modified by aeration, which indicated that these components could not be oxidized by air. Exogenous oxidized cytochrome *c*, added to the *C 117* preparation, was reduced at once in the presence of succinate.

Poky *preparation*. A study of cytochrome in this strain was made by Haskins *et al.* (1953). The high concentration of cytochrome *c* was mostly in the reduced form, since its strong α-band was only slightly intensified on addition of succinate. The bands of cytochromes *b* and *a* were not visible. Cytochrome *c* could not be oxidized by aeration.

Alkali-treated heart muscle and wild-type Neurospora *preparations*. Cytochromes *a*, *b* and *c* were present in the oxidized form and their absorption bands were hardly visible. They remained oxidized on addition of succinate, both in the absence and in the presence of exogenous cytochrome *c*; they were reduced by sodium dithionite.

Reconstruction of the succinic oxidase system. In the following experiments, the absorption bands of cytochrome, in either *C 117* or *poky* preparations, were observed after the addition of a very small amount of alkali-treated preparation from heart muscle or wild-type *Neurospora*. The amount of alkali-treated preparation was such that even on addition of sodium dithionite the absorption bands of its cytochrome were not visible under the spectroscope, and thus did not modify the spectrum of *C 117* or *poky* preparations. For instance, 0·05 ml., 10 times diluted alkali-treated heart muscle, or 4 times diluted alkali-treated wild-type *Neurospora* preparation, were added to 0·4 ml. *C 117* or *poky* preparation and 0·05 ml. 0·2 M sodium succinate in a flat-bottomed glass tube of diameter 14 mm., giving a depth of about 3 mm.

The addition of alkali-treated heart muscle or wild-type *Neurospora* preparation, in the presence of a small amount of succinate (0·02 M), to a *C 117* preparation did not modify the absorption spectrum of the latter, even after aeration. However, when exogenous oxidized cytochrome *c* was added (e.g. 0·05 ml. 2×10^{-4} M cytochrome *c* in a reaction mixture of 0·5 ml.), it was reduced at once, and intermittent aeration of the mixture led to oxidation and subsequent reduction of cytochromes *b* and *c* belonging to *C 117*, and of exogenous cytochrome *c*.

On addition of a small amount of alkali-treated heart muscle or wild-type *Neurospora* preparation to the *poky* preparation in the absence of succinate, the strong cytochrome *c* band of the latter disappeared at once, thus showing that the endogenous cytochrome *c* of the *poky* particles had been oxidized by the oxidase of heart muscle or wild-type *Neurospora* particles. In the presence of succinate alone (0·02 M), cytochrome *c* remained reduced on shaking in air, but with succinate and 0·01 M malonate, the absorption band of cytochrome *c* disappeared almost completely on aeration, and slowly came back on standing, which showed that the succinic oxidase system had been reconstructed.

The possibility that the two kinds of particles from *poky* and from alkali-treated preparation had reacted together because of the presence of soluble cytochrome *c*, extracted from the *poky* particles in the suspending buffer, was examined in the following way. A *poky* preparation was made up as usual (Tissières & Mitchell, 1954) but was washed 3 times in buffer by high-speed centrifuging, instead of once. To 5 ml. of this preparation, 0·15 ml., 10 times diluted alkali-treated heart muscle, 0·25 ml. 0·4 M succinate and 0·05 ml. M malonate, were added, and after the spectroscopic observations, the mixture was centrifuged at high speed and the clear supernatant was examined spectroscopically in a long flat-bottomed tube at a depth of 12 cm., after addition of sodium dithionite. No cytochrome *c* was visible under these conditions which allow the detection of 5×10^{-8} M cytochrome *c*. The supernatant was also examined for its ability to replace cytochrome *c* in a succinic oxidase system built up from *C 117* and alkali-treated heart muscle preparations, but it was found to be ineffective. The fact that it was ineffective showed that the supernatant did not contain any significant amount of cytochrome *c*. It is therefore not likely that the reaction between the two kinds of particles, from *poky* and alkali-treated preparations, was due to the presence of a small amount of soluble cytochrome *c*.

Manometric experiments

Effect of alkali treatment. With both sources of enzymes, heart muscle or wild-type *Neurospora*, alkali treatment abolished succinic dehydrogenase activity almost completely, while cytochrome oxidase activity retained roughly half its original value. The results of a typical experiment are presented in Table 1.

Reconstruction of the succinic oxidase system. As shown in Fig. 1, the succinic oxidase system was reconstructed by mixing two inactive preparations, one from *C 117* providing succinic dehydrogenase, the other (alkali-treated wild-type *Neurospora*) providing cytochrome oxidase. The system was only active in the presence of exogenous cytochrome *c* and the activity was proportional to the concentration of this substance. In this experiment, the oxygen uptake did not increase when 0·3 ml. *C 117* preparation was used instead of 0·2 ml., which showed that the cytochrome oxidase of the alkali-treated wild-type preparation was the limiting factor. Under these conditions, the same amount of alkali-treated wild-type *Neurospora* preparation took up about 15 % less oxygen when

Table 1. *Succinic oxidase and cytochrome oxidase activities of alkali-treated and untreated heart muscle and wild-type* Neurospora *preparations*

	$-Q_{O_2}$			
	Heart muscle preparation		Wild-type *Neurospora* preparation	
	Untreated	Treated at pH 9·4	Untreated	Treated at pH 9·2
Succinic oxidase	405	6	63	0
Cytochrome oxidase	1120	520	102	62

Succinic oxidase determinations: each flask contained 4×10^{-5} M cytochrome *c*, 0·04 M Na succinate in 0·075 M phosphate buffer, pH 7·0, and the enzyme. Total volume 3·0 ml.

Cytochrome oxidase: 4×10^{-5} M cytochrome *c*, 0·5% (w/v) Na ascorbate, 0·075 M phosphate buffer, pH 7·0, and the enzyme. Total volume 3·0 ml. Gas phase air; 35°. Values of O_2 uptake as μl./mg. fat-free dry wt./hr.

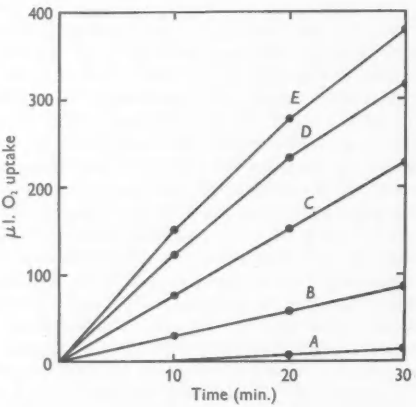

Fig. 1. Reconstruction of the succinic oxidase system by mixing, in presence of varying amounts of cytochrome *c*, two inactive preparations: one from the *Neurospora* strain *C 117* providing succinic dehydrogenase, the other, an alkali-treated wild-type preparation (AWT), providing cytochrome oxidase. The estimations of succinic oxidase activity (curves *A*, *B*, *C* and *E*) were carried out in Barcroft manometers, as described in Methods with 0·2 ml. *C 117* preparation and 0·3 ml. AWT, in the presence of the following concentrations of cytochrome *c*. Curve *A*: no cytochrome *c* added; curve *B*: 4×10^{-6} M; curve *C*: 1×10^{-5} M; curve *E*: 4×10^{-5} M. Curve *D* represents the estimation of cytochrome oxidase activity of 0·3 ml. AWT, as described in the text.

cytochrome oxidase activity in the presence of ascorbate and the same amount of cytochrome *c* was being measured.

In the experiments reported in Tables 2 and 3, the succinic oxidase system was reconstructed by mixing an alkali-treated heart muscle preparation with the *C 117* enzymes. Alkali-treated heart muscle was added in small excess, so that the *C 117* preparation was limiting. The activities of *C 117*, in terms of succinic dehydrogenase and of its ability to reconstruct the succinic oxidase system, were compared with the succinic dehydrogenase and succinic oxidase activities of the wild type (Table 3). In the case of the reconstructed system (with *C 117* and heart muscle preparation), as in the case of the wild type, the succinic dehydrogenase activity was found to be about 50–60% (depending on the concentration of methylene blue) of that of the succinic oxidase system. It should be noted that the measurement of succinic dehydrogenase activity in manometers in presence of methylene blue and cyanide is only a relative one, as it is limited by the concentration of methylene blue (Slater, 1949). The oxygen uptake given by these measurements should not therefore be compared directly with that of the succinic oxidase activity.

Poky preparations, in the presence or in the absence of added cytochrome *c*, were found to be completely ineffective in replacing the *C 117* enzyme in manometric experiments on the reconstruction of the succinic oxidase system. There is evidence that this is due to the presence in *poky* of an inhibitor. Thus, the succinic oxidase activity of either untreated wild-type or heart muscle preparations, or that of the reconstructed system with *C 117* and alkali-treated enzymes, was found to be almost completely inhibited by the addition of a small amount of *poky* preparation. That the effect of this inhibitor was not observed in spectroscopic experiments on the reconstructed system is not inconsistent with the manometric results. On the one hand, the spectroscopic method is much more sensitive than the manometric and, on the other hand, the initial reading in manometric experiments takes place several minutes after the two types of particle have been mixed, that is after the postulated inhibitor has had time to act. By contrast, the reaction observed with the spectroscope is practically instantaneous.

Table 2. *Reconstruction of the succinic oxidase system with alkali-treated heart muscle and* C 117 *preparations*

	O_2 taken up in 10 min. (μl.)			
			Succinic dehydrogenase	
	Cytochrome oxidase	Succinic oxidase	0·001 M methylene blue	0·002 M methylene blue
Alkali-treated heart muscle	172	0	0	—
C 117	—	7	75	85
Alkali-treated heart muscle + *C 117*	—	145	—	—

Cytochrome oxidase and succinic oxidase determinations as in Table 1. Succinic dehydrogenase: each flask contained 0·04 M succinate, 0·003 M cyanide and 0·001 or 0·002 M methylene blue in 0·075 M phosphate buffer pH 7·0, and the enzyme. Total volume 3·0 ml.

Table 3. *Activities of a* C 117 *preparation in the reconstruction of the succinic oxidase system, and of its succinic dehydrogenase, compared with the activities of the same enzymes in the wild type*

	$-Q_{O_2}$				
		Succinic dehydrogenase		Succinic dehydrogenase as % of succinic oxidase	
	Succinic oxidase	0·001 M methylene blue	0·002 M methylene blue	0·001 M methylene blue	0·002 M methylene blue
C 117	164 (Reconstructed system with alkali-treated heart muscle preparation)	85	98	52	60
Wild type	138	70	83	51	60

For experimental conditions see Tables 1 and 2.

DISCUSSION

The results of spectroscopic experiments show that *C 117* particles (which have normal succinic dehydrogenase, are deficient in cytochrome oxidase and lack cytochrome *c*) were unable to react with cytochrome oxidase from heart muscle or wild-type *Neurospora* preparations to reconstruct the succinic oxidase system, unless exogenous cytochrome *c* was present. *Poky* particles, on the other hand, which also have normal succinic dehydrogenase activity and are deficient in cytochrome oxidase, but contain a high concentration of cytochrome *c*, were found to react directly with cytochrome oxidase from heart muscle or wild-type preparations. Thus the α-band of reduced cytochrome *c* in *poky* preparations was not modified by aeration but it disappeared at once on addition of a small amount of heart muscle or wild-type enzyme; in the presence of 0·02 M succinate and 0·01 M malonate, cytochrome *c* was oxidized on aeration and subsequently became reduced on standing. This suggests that in the absence of a soluble component the succinic oxidase system had been reconstructed from two different types of particle. It is unlikely that the reaction was due to a small amount of cytochrome *c* extracted from the particles into the suspending buffer, as this substance was not found in the supernatant after centrifuging down the particles at high speed, and as the supernatant was ineffective in replacing cytochrome *c* in spectroscopic experiments to reconstruct the succinic oxidase system from *C 117* and alkali-treated preparations.

The fact that manometric experiments did not confirm spectroscopic observation with *poky* particles has already been explained by the inhibition that a *poky* preparation produced in the succinic oxidase system. On the other hand, the sensitivity of spectroscopic experiments performed with thick suspensions of particles is very much greater than that of manometric techniques. It is therefore not surprising that the effect noticed with the spectroscope was not detected while measuring the oxygen uptake of the succinic oxidase system. Furthermore, it is obvious that interaction of particles is a much slower process in the absence than in the presence of a soluble component, cytochrome *c*, acting as a link.

The study of the activity of the reconstructed system with *C 117* and heart muscle enzymes (Table 2) suggests that when a suitable amount of preparation from each source was used, the oxygen taken up was of the order expected from measurements of the full activity of succinic dehydrogenase in *C 117* and cytochrome oxidase in heart muscle preparations. In this connexion it is interesting to note that in Fig. 1 the amount of oxygen taken up by the reconstructed succinic oxidase system exceeded that of the alkali-treated preparation alone in presence of ascorbate.

SUMMARY

1. The succinic oxidase system was reconstructed, in presence of exogenous cytochrome *c*, from two inactive preparations, one, from the *Neurospora* strain *C 117*, providing succinic dehydrogenase, the other from alkali-treated wild-type *Neurospora* or heart muscle preparations, providing cytochrome oxidase.

2. It was found in spectroscopic experiments that particles from the *Neurospora* strain *poky* reacted directly with wild-type or heart muscle particles to reconstruct the succinic oxidase system in the absence of a soluble component.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

3. THE DISSOCIATIONS OF THE MAGNESIUM SALTS OF PHOSPHORIC ACID, GLUCOSE 1-PHOSPHORIC ACID AND GLYCEROL 2-PHOSPHORIC ACID

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A considerable body of evidence exists indicating that the magnesium salts of many divalent acids such as sulphate (Jones & Monk, 1952), malonate (Evans & Monk, 1952), orthophosphate (Greenwald, Redish & Kibrick, 1940), glycerol 1-phosphate (Tabor & Hastings, 1943) and glucose 1-phosphate (Trevelyan, Mann & Harrison, 1952) are incompletely dissociated in aqueous solution. Only in the cases of magnesium sulphate and magnesium malonate have the thermodynamic dissociations been measured at several temperatures and the thermodynamic quantities associated with the dissociations calculated. As magnesium is known to activate a number of enzyme systems concerned with phosphate metabolism, it is of interest to obtain detailed information about the dissociations of the magnesium salts of those phosphoric acids for which the thermodynamic acid dissociation constants are known.

THEORY

Jones & Monk (1952) have pointed out that the addition of magnesium or other salts of incompletely dissociated acids to cells without liquid junction of the type Pt, H_2 | HCl | AgCl, Ag will yield information concerning the dissociation of these salts. Accordingly phosphate or phosphate ester buffers containing $MgCl_2$ were examined potentiometrically in cells without liquid junction of the type

Pt, H_2 (1 atm.) | MHA (m_a). M_2A (m_b). $MgCl_2$(m_s) | AgCl. Ag,

where M is an alkali metal.

The e.m.f. of such a cell is given by

$$E = E_0 - \frac{2{\cdot}3026RT}{F} \log a_{H^+}.a_{Cl^-}. \qquad (1)$$

From this equation in terms of molalities and the approximation for the activity coefficients proposed by Güntelberg (1926), the use of which has been discussed previously (Ashby, Crook & Datta, 1954*a*) we have:

$$\log m_{H^+} = -\frac{(E-E_0)\,F}{2{\cdot}3026RT} - \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}. \qquad (2)$$

This equation, together with equation (3), the expression for the dissociation constant K_a of the acid HA^-:

$$-\log K_a = -\log \frac{m_{H^+}.m_{A^{2-}}}{m_{HA^-}} + \frac{4AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}} \qquad (3)$$

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gives

$$\log m_{A^{2-}} = \log K_a + \log m_{HA^-} + \frac{(E-E_0)\,F}{2{\cdot}3026RT} + \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}, \quad (4)$$

where $m_{HA^-} = m_a - m_{H^+}$, $m_{M^+} = m_a + 2m_b$, $m_{Cl^-} = 2m_s$, and since undissociated MgA is formed

$$m_{Mg^{2+}} = m_s - m_b + m_{H^+} + m_{A^{2-}}$$

hence $$I = m_a - m_b + 3m_s - 2m_{H^+} + 4m_{A^{2-}}. \quad (5)$$

It is apparent that equation (4) can be solved for $m_{A^{2-}}$ only by using an approximate value for I; the value of $m_{A^{2-}}$ thus obtained is used in equation (5), and the new value of I is used in equation (4) to obtain a better value of $m_{A^{2-}}$. The iteration is continued until no further change occurs in either $m_{A^{2-}}$ or I, and these final values are then used to obtain the dissociation constant of the salt

$$K_s = \frac{a_{Mg^{2+}} \cdot a_{A^{2-}}}{a_{MgA}}$$

by the equation

$$-\log K_s + \beta I = -\log m_{Mg^{2+}} - \log m_{A^{2-}} + \log m_{MgA} + \frac{8AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}} = y, \quad (6)$$

where $$m_{MgA} = m_b + m_{H^+} - m_{A^{2-}},$$

and $$m_{Mg^{2+}} = m_s - m_{MgA}.$$

The right-hand side of equation (6), designated by y, is plotted against I and gives a straight line of slope β; the intercept at $I=0$, giving $-\log K_s$, is obtained by the method of least squares.

The values of $-\log K_s$ at different temperatures vary with the absolute temperature according to the equation of Harned & Robinson (1940),

$$-\log K_s = A/T - D + CT. \quad (7)$$

The thermodynamic quantities associated with the dissociation can be calculated from the constants of equation (7).

It will have been noticed in the above treatment that the possibility of the formation of undissociated complexes of the type $MgHA^+$ or $Mg(HA)_2$ is

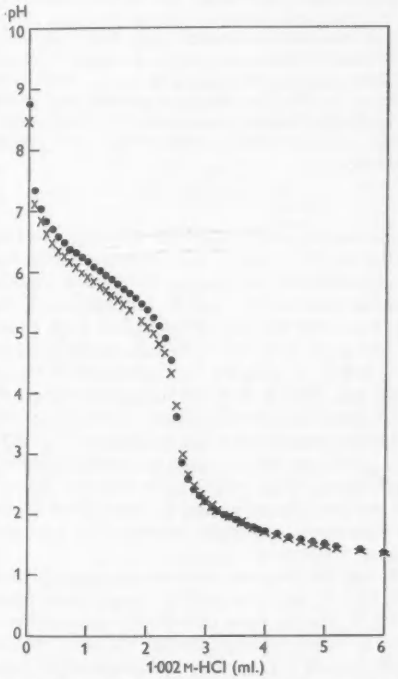

Fig. 1. Titration of dipotassium glucose 1-phosphate with HCl in the presence of NaCl and $MgCl_2$ of equal ionic strength. ●, 25 ml. 0·1 M dipotassium glucose 1-phosphate and 25 ml. 0·3 M-NaCl; ×, 25 ml. 0·1 M dipotassium glucose 1-phosphate and 25 ml. 0·1 M-$MgCl_2$.

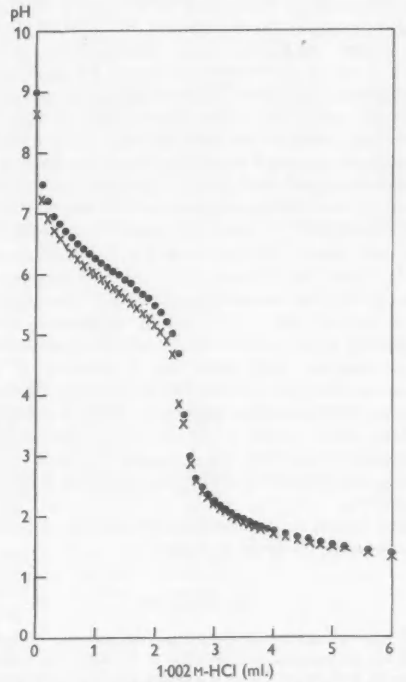

Fig. 2. Titration of disodium glycerol 2-phosphate with HCl in the presence of NaCl and $MgCl_2$ of equal ionic strength. ●, 25 ml. 0·1 M disodium glycerol 2-phosphate and 25 ml. 0·3 M-NaCl; ×, 25 ml. 0·1 M disodium glycero-2-phosphate and 25 ml. 0·1 M-$MgCl_2$.

Table 1. *Values of* $-\log K_{2a}$ *used*

Temp. (°)	Phosphoric acid	Glucose 1-phosphoric acid	Glycerol 2-phosphoric acid
10	7·254	6·5005	6·6496
20	7·213	6·4997	6·6464
30	7·190	6·5103	6·6565
40	7·182	6·5311	6·6786
50	7·184	6·5613	6·7115

Values for phosphoric acid from Bates & Acree (1943), for glucose 1-phosphoric acid from Ashby, Clarke, Crook & Datta (in preparation) and for glycerol 2-phosphoric acid from Ashby, Crook & Datta (1954*b*). Values of $-\log K_{2a}$ from our own data were used to four decimal places, though the fourth figure is not significant.

ignored. Should such complexes be formed, the titration curves of the acids in the presence of $MgCl_2$ would be displaced with respect to those in the presence of NaCl of equal ionic strength in the region of the pK_1 as well as in the region of the pK_2. Data exists showing that no such displacement occurs in the region of the pK_1 of phosphoric acid (Greenwald *et al.* 1940), and these authors conclude that no significant amount of the undissociated complexes $MgH_2PO_4^+$ or $Mg(H_2PO_4)_2$ occur. Figs. 1 and 2 show titration curves for glucose 1-phosphate and glycerol 2-phosphate in the presence of $MgCl_2$ and NaCl; from these curves it may be concluded that in the case of these acids also no significant amounts of undissociated complexes of magnesium and the singly charged acid species occur. It is of interest to note that the association of Mg^{2+} and HSO_4^- ions is considered to be negligible (Jones & Monk, 1952) and that the dissociation constant of the (Mg-H malonate)$^+$ ion pair is approximately 0·4 (Cannan & Kibrick, 1938). Ion associations between Mg^{2+}, Na^+ and Cl^- are known to be negligibly small, and in the solutions studied here m_{OH^-} was so small that the formation of the complex $Mg(OH)^+$ for which $K = 0{\cdot}0026$ (Stock & Davies, 1948) could be neglected. Tabor & Hastings (1943) have given evidence from conductance measurements that the complex formed when $MgCl_2$ and Na_2HPO_4 solutions are mixed is in fact $MgHPO_4$.

The values of the acid dissociation constants used in this work are listed in Table 1.

METHODS

The apparatus, general technique, fundamental constants and the preparation and analysis of the HCl, dipotassium glucose 1-phosphate and disodium glycerol 2-phosphate were the same as have been described previously (Ashby, Crook & Datta, 1954*a*, *b*; Ashby, Clarke, Crook & Datta, in preparation). The temperature was measured with a platinum resistance thermometer constructed in our laboratory and calibrated by the National Physical Laboratory. The resistance at the triple point of water was frequently checked.

Carbonate-free NaOH was prepared from a saturated solution of washed AnalaR NaOH pellets in water which was suitably diluted with CO_2-free conductivity water in an apparatus which allowed the dilution to be made in an atmosphere of N_2, free from CO_2. The NaOH solution was standardized on both the molar and the molal scales by volumetric and weight titrations with accurately diluted constant-boiling HCl which had itself been checked gravimetrically.

HCl, approx. 1 M, was prepared from redistilled approx. 5 M-HCl (AnalaR) and standardized by titration with the standard NaOH.

AnalaR KH_2PO_4 was recrystallized 3 times from conductivity water and dried *in vacuo* at 100°.

$MgCl_2$ solutions were prepared from AnalaR $MgCl_2, 5H_2O$ and standardized on the molal scale by gravimetric chloride estimations which agreed to within 0·1%. $MgCl_2$ solutions of known molar concentrations were prepared by weighing out the required amount of solution of known molality and making up to volume.

The titration curves (Figs. 1 and 2) were obtained with a glass electrode (Doran Instrument Co. Alkaglass electrode) and an Ag–AgCl electrode in saturated KCl as a reference using a saturated KCl bridge. The electrode assembly was standardized with 0·05 M potassium hydrogen phthalate (British Standards Institution (1950) 1647). All solutions for the titration curves were prepared in grade A volumetric apparatus and the HCl was added from a 10 ml. burette. The e.m.f. of the glass electrode assembly was measured with a Tinsley Vernier potentiometer (H. Tinsley and Co., London, S.E. 26) using an electrometer valve impedance converter.

RESULTS

The measured e.m.f.'s, together with the values of the molalities of the various ionic species and the values of y (equation 6), are given in Tables 2–4. The extrapolated values of $-\log K_a$ at infinite dilution, together with the values calculated from equation (7), are given in Table 5. The values of the constants A, C and D of equation (7), together with the minimum pK_a values and the temperatures at which they occur, are given in Table 6. Where two sets of dilutions were done for one substance, the values of A, C and D are derived from the mean values of the coefficients of the polynomials used for fitting the curves through the points. In both cases there was no significant difference between the two sets of values.

It will be observed that the standard deviations of $-\log K_a$ are considerably larger than those of $-\log K_a$ for the ester phosphates (Ashby, Crook & Datta, 1954*b*; Ashby, Clarke, Crook & Datta, in preparation), although the solutions were prepared and the e.m.f. measurements made with the same high degree of accuracy. The reason for this is that in the measurement of the acid dissociation constants the e.m.f. enters only into the value of $\log m_{H^+}$ and in the case of the first dissociation into $\log m_{HA^-}$

Table 2. *Magnesium phosphate. Molalities (m) and ionic strengths (I) of solutions, e.m.f.'s of cells (E), international volts (corrected to 1 atm. pressure of hydrogen and rounded temperatures) and extrapolation functions (y)*

m_a = molality KH_2PO_4, m_b = molality $KNaHPO_4$, m_s = molality $MgCl_2$, m_{H+} = antilog $\left[\frac{-(E-E_0)\,F}{2{\cdot}3026RT} - \log m_{Cl-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}\right]$,

$m_{A^{2-}}$ = antilog $\left[\log K_a + \log m_{HA-} - \log m_{H+} + \frac{4AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}\right]$. $I = m_a - m_b + 3m_s - 2m_{H+} + 4m_{A^{2-}}$,

$y = -\log m_{Mg^{2+}} - \log m_{A^{2-}} + \log m_{MgA} + \frac{8AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}$. In these solutions $m_{Cl-} = 2m_s$.

Set 1

	$10^3 m_a$	0·57459	1·0478	1·4015	1·7630	2·3526	2·6700	3·1520	3·7765
	$10^3 m_b$	0·46990	0·85688	1·1462	1·4418	1·9239	2·1835	2·5777	3·0884
	$10^2 m_s$	0·34802	0·63463	0·84886	1·0678	1·4249	1·6171	1·9091	2·2873
	$10^7 m_{H+}$	2	3	3	4	5	5	5	6
10°	E	0·73879	0·71896	0·70936	0·70160	0·69158	0·68721	0·68161	0·67527
	$10^3 m_{A^{2-}}$	0·30176	0·47574	0·59659	0·70983	0·87682	0·96433	1·0999	1·2588
	$10^2 I$	1·1752	2·1132	2·8107	3·5194	4·6682	5·2857	6·2246	7·4342
	y	2·6146	2·6321	2·6346	2·6427	2·6617	2·6691	2·6749	2·6896
20°	E	0·74747	0·72666	0·71654	0·70846	0·69804	0·69336	0·68766	0·68102
	$10^3 m_{A^{2-}}$	0·28966	0·45212	0·56325	0·66928	0·82586	0·90296	1·0344	1·1810
	$10^2 I$	1·1704	2·1038	2·7974	3·5032	4·6478	5·2612	6·1984	7·4031
	y	2·6701	2·6895	2·6956	2·7031	2·7209	2·7322	2·7343	2·7503
30°	E	0·75589	0·73406	0·72341	0·71498	0·70414	0·69947	0·69336	0·68644
	$10^3 m_{A^{2-}}$	0·27340	0·42215	0·52254	0·61949	0·76311	0·84019	0·95504	1·0863
	$10^2 I$	1·1638	2·0918	2·7811	3·4833	4·6227	5·2360	6·1666	7·3652
	y	2·7410	2·7609	2·7690	2·7761	2·7930	2·7985	2·8059	2·8238
40°	E	0·76365	0·74099	0·72985	0·72111	0·70986	0·70499	0·69859	0·69145
	$10^3 m_A$ –	0·25165	0·38747	0·47748	0·56578	0·69625	0·76558	0·86787	0·99041
	$10^2 I$	1·1551	2·0779	2·7630	3·4618	4·5960	5·2062	6·1318	7·3268
	y	2·8321	2·8427	2·8509	2·8565	2·8720	2·8779	2·8867	2·9018
50°	E	0·77098	0·74761	0·73593	0·72684	0·71510	0·71015	0·70331	0·69592
	$10^3 m_{A^{2-}}$	0·22862	0·35270	0·43210	0·51105	0·62652	0·69109	0·77725	0·88697
	$10^2 I$	1·1459	2·06040	2·7449	3·4399	4·5681	5·1764	6·0955	7·2855
	y	2·9275	2·9267	2·9361	2·9414	2·9578	2·9612	2·9747	2·9894

Set 2

	$10^3 m_a$	1·2137	1·6274	2·0995	2·4093	2·9098	3·4685
	$10^3 m_b$	1·0918	1·4640	1·8887	2·1674	2·6175	3·1202
	$10^2 m_s$	0·89066	1·1943	1·5407	1·7681	2·1353	2·5453
	$10^7 m_{H+}$	3	4	4	5	5	6
10°	E	0·70953	0·69956	0·69078	0·68626	0·67964	0·67342
	$10^3 m_{A^{2-}}$	0·54838	0·68407	0·82759	0·92716	1·0666	1·2143
	$10^2 I$	2·9034	3·8727	4·9742	5·6991	6·8616	8·1564
	y	2·6516	2·6619	2·6750	2·6752	2·6897	2·7054
20°	E	0·71667	0·70629	0·69710	0·69243	0·68557	0·67909
	$10^3 m_{A^{2-}}$	0·51685	0·64372	0·77626	0·87030	1·0016	1·1391
	$10^2 I$	2·8908	3·8566	4·9537	5·6764	6·8356	8·1263
	y	2·7124	2·7219	2·7362	2·7357	2·7493	2·7640
30°	E	0·72348	0·71268	0·70305	0·69826	0·69113	0·68442
	$10^3 m_{A^{2-}}$	0·47844	0·59473	0·71422	0·80234	0·92261	1·0495
	$10^2 I$	2·8755	3·8370	4·9288	5·6492	6·8040	8·0905
	y	2·7855	2·7941	2·8098	2·8078	2·8213	2·8367
40°	E	0·73000	0·71875	0·70852	0·70361	0·69629	0·68935
	$10^3 m_{A^{2-}}$	0·43850	0·54374	0·64667	0·72782	0·83891	0·95470
	$10^2 I$	2·8595	3·8166	4·9018	5·6194	6·7705	8·0525
	y	2·8632	2·8717	2·8924	2·8892	2·9005	2·9154
50°	E	0·73599	0·72430	0·71353	0·70851	0·70098	0·69375
	$10^3 m_{A^{2-}}$	0·39553	0·48948	0·57818	0·65212	0·75292	0·85535
	$10^2 I$	2·8423	3·7948	4·8744	5·5891	6·7362	8·0128
	y	2·9490	2·9571	2·9806	2·9761	2·9863	3·0022

Table 3. *Magnesium glucose* 1*-phosphate. Molalities* (m) *and ionic strengths* (I) *of solutions, e.m.f.'s of cells* (E), *international volts (corrected to* 1 *atm. pressure of hydrogen and rounded temperatures) and extrapolation functions* (y)

m_a = molality KH glucose 1-phosphate, m_b = molality K_2 glucose 1-phosphate, m_s = molality $MgCl_2$, m_{H^+} = antilog $\left[\frac{-(E-E_0)F}{2{\cdot}3026RT} - \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}\right]$,

$m_{A^{2-}}$ = antilog $\left[\log K_a + \log m_{HA^-} - \log m_{H^+} + \frac{4AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}\right]$, $I = m_a - m_b + 3m_s - 2m_{H^+} + 4m_{A^{2-}}$, $y = -\log m_{Mg^{2+}} - \log m_{A^{2-}} + \log m_{MgA} + \frac{8AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}$.

In these solutions $m_{Cl^-} = 2m_s + m_a$.

	$10^3 m_a$	0·92740	1·0624	1·2733	1·4643	1·7462	1·9342	2·3541	2·6213	3·0539	3·5574	4·0035	4·2827
	$10^3 m_b$	2·7274	3·1246	3·7448	4·3064	5·1353	5·6882	6·9231	7·7089	8·9811	10·4619	11·7739	12·5951
	$10^2 m_s$	0·43093	0·49368	0·59168	0·68041	0·81138	0·89873	1·0939	1·2180	1·4190	1·6530	1·8603	1·9900
	$10^7 m_{H^+}$	3	3	3	3	3	4	4	4	4	5	5	5
10°	E	0·72302	0·71885	0·71317	0·70882	0·70319	0·70008	0·69399	0·69060	0·68563	0·68076	0·67714	0·67498
	$10^3 m_{A^{2-}}$	2·1126	2·3757	2·7665	3·1193	3·6099	3·9597	4·7139	5·1778	5·8813	6·7170	7·5006	7·9572
	$10^2 I$	2·0505	2·3313	2·7618	3·1511	3·7137	4·0980	4·9455	5·4784	6·3221	7·3109	8·2043	8·7499
	y	2·3937	2·4024	2·4205	2·4293	2·4484	2·4473	2·4518	2·4575	2·4743	2·4842	2·4830	2·4878
20°	E	0·73363	0·72946	0·72352	0·71904	0·71306	0·70981	0·70352	0·69996	0·69472	0·68970	0·68592	0·68368
	$10^3 m_{A^{2-}}$	2·0393	2·3081	2·6820	3·0278	3·4831	3·8169	4·5486	4·9867	5·6442	6·4548	7·2004	7·6393
	$10^2 I$	2·0212	2·3043	2·7279	3·1145	3·6630	4·0409	4·8794	5·4020	6·2273	7·2060	8·0842	8·6227
	y	2·4720	2·4659	2·4843	2·4889	2·5144	2·5140	2·5155	2·5227	2·5424	2·5496	2·5495	2·5540
30°	E	0·74407	0·73986	0·73364	0·72885	0·72270	0·71926	0·71273	0·70906	0·70353	0·69834	0·69440	0·69207
	$10^3 m_{A^{2-}}$	1·9592	2·2271	2·5807	2·8957	3·3372	3·6460	4·3425	4·7643	5·3703	6·1442	6·8480	7·2628
	$10^2 I$	1·9892	2·2718	2·6874	3·0617	3·6047	3·9725	4·7970	5·3130	6·1177	7·0818	7·9432	8·4721
	y	2·5526	2·5376	2·5565	2·5681	2·5871	2·5900	2·5906	2·5958	2·6184	2·6241	2·6250	2·6294
40°	E	0·75467	0·74993	0·74350	0·73840	0·73205	0·72886	0·72206	0·71825	0·71208	0·70657	0·70253	0·70001
	$10^3 m_{A^{2-}}$	1·9033	2·1312	2·4703	2·7563	3·1780	3·5231	4·1896	4·5946	5·0884	5·7906	6·4635	6·8257
	$10^2 I$	1·9668	2·2335	2·6433	3·0059	3·5410	3·9234	4·7358	5·2452	6·0050	6·9404	7·7894	8·2973
	y	2·6101	2·6178	2·6324	2·6492	2·6643	2·6479	2·6499	2·6551	2·6962	2·7071	2·7060	2·7146
50°	E	0·76438	0·75971	0·75305	0·74748	0·74116	0·73731	0·73030	0·72647	0·72042	0·71465	0·71040	0·70763
	$10^3 m_{A^{2-}}$	1·7812	2·0120	2·3306	2·5712	2·9924	3·2481	3·8655	4·2570	4·7748	5·4186	6·0321	6·3301
	$10^2 I$	1·9180	2·1858	2·5874	2·9319	3·4667	3·8134	4·6062	5·1102	5·8795	6·7916	7·6169	8·0990
	y	2·7194	2·7117	2·7228	2·7502	2·7510	2·7592	2·7581	2·7574	2·7812	2·7937	2·7952	2·8093

Table 4. *Magnesium glycerol 2-phosphate. Molalities (m) and ionic strengths (I) of solutions, e.m.f.'s of cells (E), international volts (corrected to 1 atm. pressure of hydrogen and rounded temperatures) and extrapolation functions (y)*

m_a = molality NaH glycerol 2-phosphate, m_b = molality Na_2 glycerol 2-phosphate, m_s = molality $MgCl_2$,
m_{H^+} = antilog $\left[\frac{-(E-E_0)F}{2{\cdot}3026RT} - \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}\right]$, $m_{A^{2-}}$ = antilog $\left[\log K_a + \log m_{HA^-} - \log m_{H^+} + \frac{4AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}\right]$,
$I = m_a - m_b + 3m_s - 2m_{H^+} + 4m_{A^{2-}}$, $y = -\log m_{Mg^{2+}} - \log m_{A^{2-}} + \log m_{MgA} + \frac{8AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}}$. In these solutions $m_{Cl^-} = 2m_s + m_a$.

		Set 1				Set 2			
	$10^3 m_a$	1·0899	1·9658	3·2172	4·2023	1·2938	2·4568	3·3851	4·5928
	$10^3 m_b$	3·1331	5·6508	9·2482	12·0799	2·9396	5·5824	7·6917	10·4361
	$10^2 m_s$	0·48918	0·88227	1·4439	1·8861	0·49039	0·93123	1·2831	1·7409
	$10^7 m_{H^+}$	2	3	3·5	4	2·5	3·5	4·5	5
10°	E	0·72678	0·70839	0·69229	0·68426	0·72050	0·70050	0·68990	0·68039
	$10^3 m_{A^{2-}}$	2·3786	3·9470	5·8752	7·6355	2·2280	3·8702	5·0062	6·4864
	$10^2 I$	2·3236	4·0536	6·4004	8·3440	2·3271	4·2748	5·7595	7·6921
	y	2·4096	2·4479	2·5162	2·4959	2·4073	2·4449	2·4913	2·5171
20°	E	0·73735	0·71823	0·70144	0·69304	0·73081	0·70998	0·69889	0·68894
	$10^3 m_{A^{2-}}$	2·2934	3·7971	5·6303	7·2954	2·1458	3·7118	4·7816	6·1734
	$10^2 I$	2·2895	3·9937	6·3025	8·2080	2·2942	4·2114	5·6697	7·5669
	y	2·4866	2·5178	2·5837	2·5677	2·4855	2·5182	2·5640	2·5912
30°	E	0·74783	0·72787	0·71044	0·70165	0·74098	0·71929	0·70783	0·69753
	$10^3 m_{A^{2-}}$	2·1987	3·6173	5·3553	6·9148	2·0501	3·5301	4·5527	5·8813
	$10^2 I$	2·2517	3·9217	6·1925	8·0558	2·2559	4·1388	5·5781	7·4500
	y	2·5675	2·5977	2·6576	2·6460	2·5708	2·5986	2·6375	2·6616
40°	E	0·75813	0·73732	0·71919	0·71000	0·75100	0·72839	0·71648	0·70569
	$10^3 m_{A^{2-}}$	2·0948	3·4250	5·0527	6·5013	1·9492	3·3350	4·2935	5·5182
	$10^2 I$	2·2101	3·8448	6·0715	7·8904	2·2156	4·0608	5·4744	7·3048
	y	2·6521	2·6809	2·7376	2·7301	2·6568	2·6828	2·7190	2·7455
50°	E	0·76828	0·74658	0·72773	0·71821	0·76068	0·73726	0·72478	0·71395
	$10^3 m_{A^{2-}}$	1·9727	3·2021	4·7058	6·0508	1·8198	3·1072	3·9749	5·1738
	$10^2 I$	2·1613	3·7557	5·9327	7·7103	2·1638	3·9696	5·3470	7·1670
	y	2·7464	2·7741	2·8277	2·8208	2·7603	2·7777	2·8152	2·8270

(equations 2 and 3), while in the present case the e.m.f. measurement is used to estimate $m_{A^{2-}}$, which is then used to estimate $m_{Mg^{2+}}$ and m_{MgA}, and it is obvious from equation (6) that any error in the evaluation of $m_{A^{2-}}$ will be magnified in $-\log K_s$. Further, $m_{A^{2-}}$ enters as an important term in the ionic strength (equation 5). Thus it is to be expected that for a given accuracy in the experimental part of this work the errors of the values of $-\log K_s$ will be an order of magnitude greater than errors in $-\log K_a$.

In Table 8 are collected values for the dissociation constants of the magnesium salts studied in this work and some others which may be of interest to biochemists. The thermodynamic quantities associated with the dissociations of these magnesium salts are given in Table 7. The methods of calculating the standard deviations of $-\log K_s$ and of the thermodynamic quantities have been described previously (Please, 1954).

DISCUSSION

The results given in Table 8 show that the magnesium salts of glucose 1-phosphoric acid and glycerol 2-phosphoric acid are very similar in their dissociation constants; $MgHPO_4$ has a somewhat lower dissociation constant. Magnesium sulphate and magnesium malonate dissociate to much the same extent as the three magnesium phosphates. The values of $-\log K_s$ of both $MgHPO_4$ and magnesium glucose 1-phosphate calculated from the results of Trevelyan *et al.* (1952) are larger than those found in this work. This may be due in part to their use of magnesium sulphate as a source of Mg^{2+}, the erroneous assumption being made that this salt is fully dissociated. From the known value of the dissociation constant of magnesium sulphate, it may be calculated that in the experiments of Trevelyan *et al.* (1952), where the concentration of

Table 5. *Observed and calculated values of* $-\log K$ *at rounded temperatures together with values of '* β*' the slope of the extrapolation*

Calculated values are from $-\log K_s = A/T - D + CT$, where T is the absolute temperature and A, C and D are constants fitted to the observed values by the method of least squares. The standard deviation, σ, of the calculated values is derived as described by Please (1954). Values of $-\log K_s$ (obs.) are given to four decimal places, as these figures were used to calculate the values of the constants A, C and D, and $-\log K_s$ (calc.).

	Magnesium phosphate				Magnesium glucose 1-phosphate				Magnesium glycerol 2-phosphate			
	Set 1		Set 2						Set 1		Set 2	
Temp. (°)	$-\log K_s$ (obs.)	β	$-\log K_s$ (obs.)	β	$-\log K_s$ (obs.)	β	$-\log K_s$ (calc.)		$-\log K_s$ (obs.)	β	$-\log K_s$ (obs.)	β	$-\log K_s$ (calc.)
10	2·6033	1·18	2·6229	0·99	2·612	2·3839	1·31	2·386	2·3823	1·61	2·3587	2·12	2·371
20	2·6601	1·25	2·6851	0·96	2·674	2·4507	1·31	2·448	2·4582	1·55	2·4368	2·09	2·447
30	2·7317	1·26	2·7583	0·95	2·745	2·5262	1·33	2·520	2·5400	1·51	2·5289	1·82	2·532
37	—	—	—	—	2·801	—	—	2·575	—	—	—	—	2·595
40	2·8192	1·12	2·8367	0·97	2·826	2·5892	1·56	2·600	2·6241	1·52	2·6152	1·80	2·624
50	2·9081	1·08	2·9225	0·99	2·916	2·6941	1·42	2·690	2·7198	1·48	2·7283	1·43	2·723
					$\sigma = \pm 0{\cdot}003$			$\sigma = \pm 0{\cdot}007$					$\sigma = \pm 0{\cdot}015$

Table 6. *Constants of the equation*

$$-\log K_s = A/T - D + CT,$$

where T is the absolute temperature, together with the minimum $-\log K_s$ *values and the temperature at which these occur*

Constant	Magnesium phosphate	Magnesium glucose 1-phosphate	Magnesium glycerol 2-phosphate
A	1305	1288	1058
D	8·185	8·298	7·131
C	0·02186	0·02166	0·02036
$-\log K_{s\,(\text{min.})}$	2·50	2·27	2·15
$T_{(\text{min.})}$	245° K	240° K	230° K

magnesium sulphate was 0·03 M, between one-quarter and one-third of the magnesium sulphate would not have been dissociated.

The value for $-\log K_s$ of $MgHPO_4$ of Tabor & Hastings (1943) by a conductometric method is in good agreement with the value found by the potentiometric method, and the value of $-\log K_s$ these authors give for magnesium glycerol 1-phosphate is close to the potentiometric value for magnesium glycerol 2-phosphate.

The values of $-\log K_s$ values for $MgATP^{2-}$, $MgADP^-$ and magnesium phosphopyruvate of Burton & Krebs (1953) are higher than those for other magnesium phosphates. It is to be noted that these values are extremely sensitive to ionic strength and the values of $-\log K_s$ at infinite dilution calculated from the Güntelberg equation for the activity coefficients emphasize both the lower degree of dissociation and the sensitivity of $-\log K_s$ values to ionic strength.

A comparison of the thermodynamic data for the dissociation of magnesium sulphate, magnesium malonate and the three magnesium phosphates given in Table 7, shows that the values of ΔG^0, $-\Delta H^0$ and $-\Delta S^0$ are very similar except for the values of $-\Delta H^0$ and $-\Delta S^0$ for magnesium sulphate which are considerably larger than those for the other compounds. ΔG^0 at 20° for the dissociation of $MgATP^{2-}$, $MgADP^-$ and magnesium phosphopyruvate are 7·27, 5·96 and 5·19 kcal./mole respectively, which are markedly higher than those for the other magnesium salts.

The importance of complex formation between magnesium and phosphates in studies of equilibria in biological systems has been discussed by Trevelyan *et al.* (1952) and Burton & Krebs (1953). It is also of interest to note that magnesium ions activate phosphatases in the pH region where divalent ester phosphate ions predominate (Roche, 1950), and it is apparently only with such divalent ester phosphate ions that magnesium forms undissociated complexes.

Table 7. *Thermodynamic quantities associated with the dissociations of the magnesium salts of phosphoric acid, glucose 1-phosphoric acid, glycerol 2-phosphoric acid, sulphuric acid and malonic acid*

σ = the standard error. The values for magnesium sulphate from Jones & Monk (1952), and for magnesium malonate from Evans & Monk (1952).

	Temp. (°)	ΔG^0 Cal.	ΔG^0 Joule	ΔH^0 Cal.	ΔH^0 Joule	ΔS^0 Cal. deg.$^{-1}$	ΔS^0 Joule deg.$^{-1}$	ΔC_P^0 Cal. deg.$^{-1}$	ΔC_P^0 Joule deg.$^{-1}$
Magnesium phosphate	25	3695	15 460	−2917	−12 200	−22·2	−92·9	−60	−251
		σ3·1	13	42	176	0·15	0·6	7	29
	37	3974	16 620	−3640	−15 200	−24·6	−102·9	−62	−259
		σ3·1	13	43	180	0·17	0·7	7·3	31
Magnesium glucose 1-phosphate	25	3382	14 150	−2913	−12 190	−21·1	−88·3	−59	−247
		σ7·2	30	96	402	1·0	4·2	16	67
	37	3654	15 290	−3640	−15 200	−23·5	−98	−61·5	−257
		σ7·3	31	98	410	1·0	4·2	17	71
Magnesium glycerol 2-phosphate	25	3392	14 190	−3437	−14 380	−22·9	−95·8	−56	−234
		σ14	59	197	824	0·7	2·9	33	138
	37	3680	15 390	−4110	−17 190	−25·1	−105	−58	−243
		σ15	63	200	840	0·7	2·9	34	140
Magnesium sulphate	25	3200	13 400	−5700	−24 000	−31·0	−130	—	—
Magnesium malonate	25	3900	16 300	−3200	−13 400	−23·9	−100	—	—

Table 8. *The dissociation constants of a number of magnesium salts given in the literature together with their calculated thermodynamic dissociation constants and the method by which they were calculated*

Salt	$-\log K_s$ Quoted value	Calculated value $I=0$	This paper $I=0$	Quoted value of I	Temp. (°)	Remarks	Author
Magnesium phosphate	1·52	2·99	2·745	0·2	30	Calc. by Güntelberg eqn. $\beta=1$	1
	2·87	—	2·81	0	38	By conductivity	2
	2·50	—	2·67 (20°)	0	Room temp.	From author's equation	3
Magnesium glucose 1-phosphate	1·29	2·83	2·52	0·2	30	Calc. by Güntelberg eqn. $\beta=1·33$	1
Magnesium glycerol 2-phosphate	—	—	2·60	—	38	—	2
Magnesium glycerol 1-phosphate	1·28	2·60	—	0·16	38	By conductivity. Calc. by Güntelberg eqn. $\beta=1·65$	2
Magnesium ATP^{2-}	2·92	5·42 (20°)	—	0·2	—	Calc. by Güntelberg eqn. $\beta=0$	4
Magnesium ADP^{-}	2·57	4·44 (20°)	—	0·2	—	Calc. by Güntelberg eqn. $\beta=0$	4
Magnesium phospho-pyruvate	2·0	3·87 (20°)	—	0·2	—	Calc. by Güntelberg eqn. $\beta=0$	4
Magnesium malonate	2·936	—	—	0	37	From authors' equation	6
Magnesium sulphate	2·516	—	—	0	37	Graphically from authors' data	5

1, Trevelyan, Mann & Harrison (1952); 2, Tabor & Hastings (1943); 3, Greenwald, Redish & Kibrick (1940); 4, Burton & Krebs (1953); 5, Jones & Monk (1952); 6, Evans & Monk (1952).

SUMMARY

1. The thermodynamic dissociation constants of magnesium phosphate, magnesium glucose 1-phosphate and magnesium glycerol 2-phosphate on the molal scale have been measured at 10° intervals from 10° to 50° using hydrogen and silver–silver chloride electrodes in cells without liquid junction.

2. From these measurements have been calculated the standard free energy, heat content, entropy and heat capacity for the ionizations at 25 and 37°.

3. The magnitude and sources of error are discussed.

4. Sufficient information has been given for the

dissociations of these salts to be considered quantitatively in any kinetic or other study of phosphate, glucose 1-phosphate or glycerol 2-phosphate in the presence of magnesium ions.

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The Estimation of Lactic Acid using Ceric Sulphate

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Gordon & Quastel (1939) showed that ceric sulphate oxidizes lactic acid according to the following equation:

$$CH_3.CHOH.COOH + 2Ce^{4+} \rightarrow CH_3.CHO + CO_2 + 2H^+ + 2Ce^{3+},$$

and they made this reaction the basis of a method for the estimation of lactic acid. The reaction was carried out at 50° and the acetaldehyde so formed was removed by a stream of nitrogen, trapped in bisulphite and estimated iodometrically in the usual way. The advantages claimed for this procedure were simplicity and the fact that very few compounds gave rise to volatile aldehydes under the experimental conditions recommended by the authors. Glucose in particular did not interfere and a preliminary treatment of glucose-containing test solutions with the copper–lime reagent was not considered necessary.

In its essentials the method devised by Long (1946) was similar to that of Gordon & Quastel (1939). He showed, however, that ceric sulphate oxidizes acetaldehyde to acetic acid; but in spite of this he was able to obtain good yields of acetaldehyde from lactic acid by aerating very rapidly, thus removing the acetaldehyde almost as fast as it was formed. The aeration rate recommended was 500 ml. air/min. as compared with the three to four bubbles nitrogen/sec. advised by Gordon & Quastel.

Winnick (1942) carried out the reaction in Conway vessels with bisulphite in the central chamber to trap the acetaldehyde and incubated for 2 hr. at 50°. His method was modified by Conway (1950), who carried out the oxidation in stoppered test tubes. After 30 min. incubation at 37°, he transferred 1 ml. samples of the reaction mixture to diffusion vessels where the acetaldehyde was trapped in bisulphite placed in the centre chamber.

In view of the widely divergent conditions recommended by the various authors, it seemed desirable, before using any of the ceric sulphate methods, to examine the various steps in the procedure further. The results of these investigations are given below.

EXPERIMENTAL AND RESULTS

Factors influencing the oxidation of lactic acid by ceric sulphate

Effect of ceric sulphate concentration and temperature on the velocity of the reaction. Since carbon dioxide is one of the products of the reaction, Warburg manometers were used to study the effect of both temperature and concentration of ceric sulphate on the velocity of the reaction. The reaction was carried out in N sulphuric acid and 0·2 ml. lithium lactate solution in N sulphuric acid was added from the side

bulb when thermal equilibrium had been attained. In Fig. 1 are plotted the half times for the reaction at 22, 40 and 53°. It will be seen that the concentration of ceric sulphate has a pronounced effect and that at 53° the half time is less than 5 min. even with the most dilute solution of ceric sulphate tested.

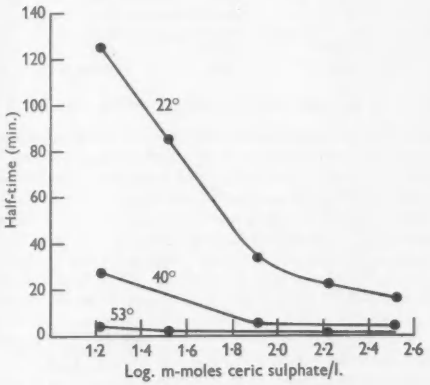

Fig. 1. Effect of temperature and ceric sulphate concentration on the velocity of oxidation of lactic acid. Experiments were carried out in Warburg manometers. 11·1 μ-moles lithium lactate in 0·2 ml. N-H_2SO_4 were added from side bulb at zero time; main compartment contained 2·8 ml. ceric sulphate in sulphuric acid; gas phase, air.

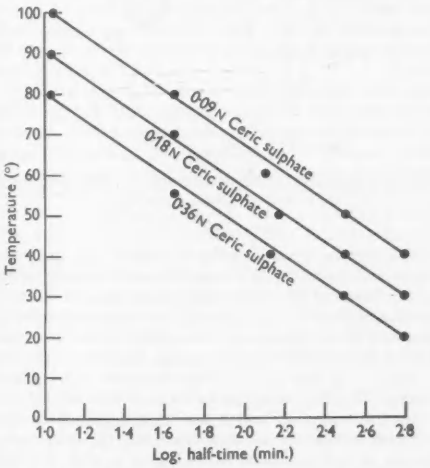

Fig. 2. Effect of temperature and ceric sulphate concentration on the velocity of oxidation of acetaldehyde. Experiments were carried out in an apparatus similar to that of Long (1946). Volume of reactants, 10 ml.; each tube contained 26·2 μmoles acetaldehyde; concentration of sulphuric acid, N. Reaction was stopped by the addition of excess of ferrous sulphate and residual acetaldehyde aerated into bisulphite and estimated iodometrically.

Oxidation of acetaldehyde by ceric sulphate. Acetaldehyde and ceric sulphate were incubated in Long's apparatus at various temperatures and, at suitable time intervals, the residual ceric sulphate was reduced to the cerous form by addition of excess of ferrous sulphate. The acetaldehyde remaining was then transferred by aeration to bisulphite solution and estimated iodometrically. The test solution contained 26·2 μmoles acetaldehyde, and the total volume including the ceric sulphate solution was 10 ml. The final concentration of sulphuric acid was normal in all cases. The results are given in Fig. 2 in which the log of the half-time of acetaldehyde destruction is plotted against temperature for three different concentrations of ceric sulphate. It will be seen that the rate of oxidation of acetaldehyde is greater the higher the temperature and the greater the ceric sulphate concentration.

Removal of acetaldehyde by aeration. The effects of temperature and velocity of aeration on the rate of removal of acetaldehyde were next examined. These experiments were conducted in an apparatus of the form and dimensions recommended by Long (1946). The volume of the reaction mixture was 10 ml. containing 20 μmoles of acetaldehyde in N sulphuric acid. Air was blown through the mixture and the acetaldehyde was trapped in bisulphite. The rate of air flow was measured with a flow meter. After suitable intervals of time, aeration was stopped and the acetaldehyde trapped in the bisulphite determined iodometrically. The experiments were carried out with flows of 200–1000 ml. air/min.

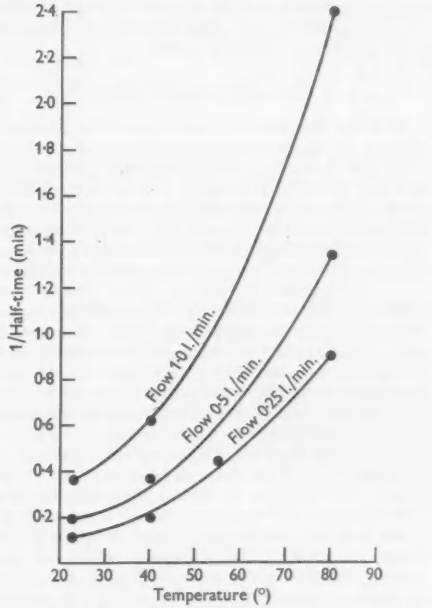

Fig. 3. Effect of rate of air flow and temperature on the rate of removal of acetaldehyde. Experiments were carried out in apparatus similar to that of Long (1946). Each apparatus contained 20 μmoles acetaldehyde in 10 ml. N sulphuric acid. Acetaldehyde was trapped in bisulphite and estimated iodometrically.

Table 1. *Recovery of lactic acid by aeration method*

Each reaction tube contained a total volume of 10 ml. mixture which was normal with respect to H_2SO_4 and contained 500 μequiv. ceric sulphate; $T = 60°$, aeration rate = 500 ml./min., $t = 45$ min.

Expt. no.	1	2	3	4
Zinc lactate added (as μmoles lactic acid)	5·65	11·3	22·4	112·9
No. of blanks	2	2	2	2
No. of estimations	4	4	4	4
Mean blank value (μmoles lactic acid)	1·13	0·87	1·37	0·97
Mean recovery corrected for blanks (μmoles lactic acid)	5·39	11·2	22·2	109·6
Range	5·00–5·69	10·88–11·91	21·11–22·40	107·9–110·7

and at temperatures from 20 to 80°. The results are given in Fig. 3. Over the range studied the rate of removal of acetaldehyde was found to be approximately proportional to (air flow)$^{0·75}$ and to the vapour pressure of acetaldehyde which would exist over pure acetaldehyde at the temperature of the experiment.

Working conditions for the aeration method. The results of the above experiments show that, for a given air flow, increasing either the ceric sulphate concentration or the temperature will diminish the recovery of acetaldehyde. These experiments have enabled us to define working conditions which permit a good recovery of acetaldehyde from lactic acid. We recommend an initial concentration of ceric sulphate of 0·05 N, a temperature of 50–60°, an aeration rate of 500–600 ml. air/min./reaction tube and an aeration time of at least 45 min. Representative recoveries using these conditions are given in Table 1.

Steam distillation method

Although the aeration method gives satisfactory recoveries, the blanks were substantial, due to the contamination of the laboratory air with carbonyl compounds. We therefore examined alternative ways of carrying out the reaction. The results described above show that to obtain the greatest rates of formation and the smallest losses of acetaldehyde, it is desirable to work at as high a temperature as is compatible with the maximum usable rate of aeration and the minimum concentration of ceric sulphate. Steam distillation seemed to offer the best possibility, and preliminary experiments in which the reaction was carried out in the apparatus of Markham (1942) indicated that at 100° the reaction was complete in a very short time and that the acetaldehyde could be distilled over into bisulphite in about 10 ml. of distillate. However, losses were encountered and these we considered to be due to the fact that all the ceric sulphate had to be added at once, with the result that, at the temperature of the mixture, the amount of ceric sulphate present was sufficient to oxidize significant amounts of acetaldehyde. We therefore designed an apparatus in which it was possible to add the ceric sulphate dropwise whilst the steam was passing. The apparatus, which has proved entirely satisfactory, is shown in Fig. 4. The following reagents are required: (i) 0·05 N ceric sulphate in N sulphuric acid prepared from a stock solution of 0·5 N ceric sulphate in N sulphuric acid. The stock ceric sulphate is standardized with ferrous ammonium sulphate. We have found ceric sulphate (pure), Hopkin & Williams, to be satisfactory. (ii) 10 N sulphuric acid. (iii) 0·5 % (w/v) sodium bisulphite solution. (iv) 0·1 N, 0·01 N and 0·005 N iodine solution.

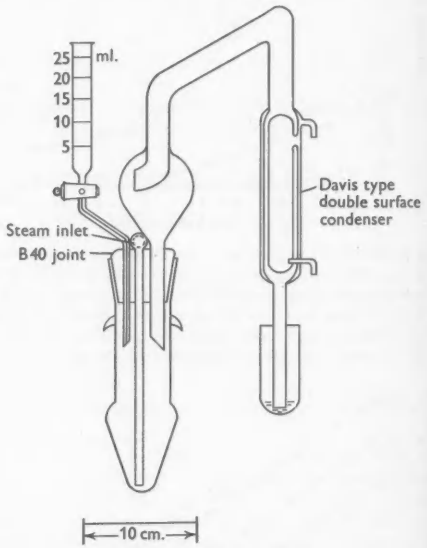

Fig. 4. Apparatus for the estimation of lactic acid by the steam-distillation method.

The sample containing lactic acid, which preferably should not exceed 5 ml., is measured into the reaction flask and sufficient of the 10 N sulphuric acid added to make the concentration of this acid normal. The reaction flask is then attached to the apparatus. The receiving tube which contains 2 ml. of the 0·5 % (w/v) sodium bisulphite is attached in such a way that the tip of the condenser dips below the surface of the bisulphite solution. The micro-burner heating the reaction flask is now turned on and the flame adjusted so that the solution just boils. The steam is then turned on and the steam flow adjusted so that 15–20 ml./min. of distillate is collected (this rapid distillation rate is essential and necessitates the use of an efficient double surface condenser); the 0·05 N ceric sulphate is then run dropwise into the reaction flask at such a rate that each drop is decolorized before the next drop goes in. When a permanent yellow colour is obtained, indicating that an excess of ceric sulphate is present, further ceric sulphate up to a total of 5 ml. is rapidly added. The only critical feature of the

Table 2. *Recovery of lactic acid by the steam distillation method*

In Expt. 1, 0·002574 N-I_2 was used for final titration; blank titration 0·08 ml. 0·002574 N-I_2, and the results corrected for this amount. In remaining experiments 0·01287 N-I_2 used and the blank was too small to be measured.

Expt. no. ...	1	2	3	4
No. of estimations	6	6	6	6
Lactic acid taken (μmoles)	2·17	10·85	21·70	32·55
Mean lactic acid recovered (μmoles)	2·14	10·64	21·13	31·82
S.E.	±0·02	±0·05	±0·03	±0·10
Range	2·08–2·16	10·60–10·73	21·07–21·14	31·61–31·87

estimation is the dropwise addition of ceric sulphate. When 15 ml. of distillate have been collected the receiver is lowered and distillation is continued until 20 ml. has been collected. The steam is then discontinued, the microburner turned out and the receiver placed in an ice-water bath to cool. While it is cooling, the reaction flask is disconnected and thoroughly washed out with distilled water. The still head is also carefully washed to remove traces of ceric sulphate solution, which, if left, could bring about the premature oxidation of part of the lactic acid in the next sample to be analysed.

The acetaldehyde is then estimated in the usual way, using solid $NaHCO_3$ to destroy the aldehyde bisulphite compound. Like Friedemann & Graeser (1933) we have found that it is important to cool the mixture thoroughly before the final titration; the temperature should be 4–5°. The results of a series of recovery experiments are given in Table 2.

Under the conditions of this method, glucose gives rise to carbonyl compounds, and we have found it essential to treat the test solution with the copper–lime reagent; for this purpose we use 1 ml. 20% (w/v) $CuSO_4$, $5H_2O$/10 ml. test solution and a spatula full of solid $Ca(OH)_2$. This reagent will also effectively deproteinize bacterial suspensions and rumen liquor.

Trichloroacetic acid cannot be used, since under the conditions of the estimation it is oxidized with formation of such large volumes of gas that frothing is uncontrollable. The various methods for the precipitation of proteins which involve the use of tungstic acid have however proved entirely satisfactory. We have also found that perchloric acid (Neuberg, Strauss & Lipkin, 1944) is very satisfactory where an acid precipitation is required. If perchloric acid treatment is followed by treatment with the copper–lime reagent we have found it convenient to add the copper solution followed by 2 N-KOH until a slight trace of cupric hydroxide is formed, at which point the lime is added. In this way the perchloric acid is removed as the insoluble potassium salt. The removal of perchloric acid is not essential.

Protein hydrolysates, such as are used in bacteriological media cause some interference, but no carbonyl compounds were produced from ethanol, from citric acid or from malic acid.

The results of an experiment in which the lactic acid, formed from malic acid by *Lactobacillus arabinosus* under the conditions described by Nossal (1951), was estimated both manometrically and by the steam distillation method after treatment of the mixture with copper–lime, are given in Table 3. Table 4 shows the results obtained when a sample of blood was analysed in triplicate by the steam distillation method.

Table 3. *Estimation of lactic acid formed from* DL-*malic acid by the action of the malic decarboxylase of* Lactobacillus arabinosus

The malic decarboxylase converts L-malic acid quantitatively into lactic acid and CO_2, and the amount of CO_2 formed is thus a measure of the amount of lactic acid. In this experiment a solution of DL-malic acid was treated with the decarboxylase preparation according to the procedure of Nossal (1951), and at the end of the experiment the cup contents were transferred quantitatively to a measuring cylinder, treated with the copper–lime reagent, made up to 10 ml. and centrifuged; samples of the supernatant solution were used for the estimation of lactic acid by the steam distillation method. Strength of iodine, 0·002754 N.

	CO_2 (μmoles)	Lactic acid (μmoles)
Manometric method	9·37	9·37
Steam distillation method		
(*a*)	—	9·35
(*b*)	—	8·92

Table 4. *Estimation of lactic acid in defibrinated sheep's blood by the steam distillation method*

Three 2 ml. samples of defibrinated sheep's blood were taken and 7 ml. of distilled water added to each followed by 2 ml. 15% (w/v) perchloric acid. Precipitates were centrifuged off and supernatants filtered through cotton wool into graduated centrifuge tubes; 0·5 ml. 20% (w/v) $CuSO_4$, $5H_2O$ was added and 40% (w/v) KOH added dropwise until a slight permanent blue precipitate obtained, a spatula full of solid $Ca(OH)_2$ (approx. 1 g.) added, the contents of the tubes were mixed and the volumes noted. After 30 min. tubes were centrifuged and 5 ml. samples taken for analysis.

Sample	Lactic acid μmoles/100 ml. blood
1	193
2	197
3	200

DISCUSSION

Our observations on the reaction between ceric sulphate and lactic acid confirm and amplify those published by Long (1946). Significant amounts of the acetaldehyde produced are oxidized to acetic acid if the former is not rapidly removed from the reaction mixture. The rate of destruction of acetaldehyde is related to the concentration of ceric

sulphate in the reaction mixture and to the temperature. Consequently, to obtain maximum recovery of acetaldehyde, the concentration of ceric sulphate should be such as to ensure only a slight excess at the end of the reaction. The rate of aeration should be the maximum possible. From our results it would appear that the concentration of ceric sulphate recommended by Long (1946) is somewhat too high.

In view of our findings it is difficult to understand how a quantitative conversion of lactic acid to acetaldehyde can be obtained by the procedure of Winnick (1942). This author recommends a mixture of 3 ml. sample and 1 ml. saturated ceric sulphate and the reaction is carried out in Conway vessels and thus relies upon diffusion to remove the acetaldehyde. Since the purity of his ceric sulphate is not stated we can only guess at the final concentration, but it would seem to be in the region of 0·2N. Likewise the modification of Winnick's procedure described by Conway (1950) is open to serious criticism in the light of the observations of both Long (1946) and ourselves. In this method the reaction is carried out in stoppered test tubes, under conditions somewhat similar to those used both by Long and ourselves to study the oxidation of acetaldehyde by ceric sulphate.

It is possible to remove the acetaldehyde formed from lactic acid, almost quantitatively, either by aeration or by steam distillation, and we have examined both procedures. We prefer the steam distillation method for the following reasons. The blanks are negligible save when using 0·00257N iodine; but even here they are small and are equivalent to about 0·1 μmole lactic acid. The method is extremely rapid, one distillation taking little more than a minute to perform. The end point is sharper because the final volume in which the titration is carried out is only 30 ml. and this is particularly important when small amounts of lactic acid are to be estimated. The advantages of this ceric sulphate method over the permanganate methods, described by Friedemann, Cotonio & Shaffer (1927), Friedemann & Kendall (1929), and Friedemann & Graeser (1933) are speed and the small volume in which the titration is carried out.

SUMMARY

1. The effect of ceric sulphate concentration and temperature on the oxidation of lactic acid by ceric sulphate have been investigated.

2. The effects of temperature and rate of aeration on the removal of acetaldehyde from solutions have been investigated.

3. Conditions for the estimation of lactic acid with ceric sulphate using the aeration method are described.

4. A new method for the estimation of lactic acid using ceric sulphate and the removal of acetaldehyde by steam distillation is described.

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Active Transport of Sodium Ions from the Yeast Cell

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It has been shown (Conway & Hingerty, 1948) that sodium ions which had entered muscle extensively in rats on a potassium-free diet were actively extruded on changing the animals to a potassium-rich diet. The mean half period of extrusion, even with the plasma potassium above the normal level within 24 hr. after the change, was about 3 days. Very recently, Desmedt (1953) has described a more rapid net excretion of sodium ions from the isolated frog sartorius in which sodium ions had entered extensively after immersion in a potassium-free Ringer solution in the manner described by Steinbach (1951). To demonstrate the excretion, companion muscles which were similarly treated

were re-immersed in a fluid with composition similar to that of the inorganic content of frog blood plasma (Boyle & Conway, 1941).

In the present paper, active transport of sodium ions from the yeast cell is studied. A series of experiments is described which show the surprising fact that not only is yeast capable of actively and specifically extruding sodium ions but that the rate of excretion is much more rapid than for rat muscle, the half period (under suitable conditions) being about 35 min. at 37° or about 100 min. at room temperature. The yeast experiments have been carried out on a 'sodium-yeast' (Na-yeast) produced as described by Conway & Moore, 1954.

It is considered that the evidence presented is much in favour of active transport of sodium ions by a redox-pump mechanism (Conway, 1950*b*, 1952*a*) with direct utilization of electron energy.

A preliminary account of some of the results described here was presented at the Ciba Conference on Water Metabolism (Conway, 1952*b*).

METHODS

Isotope experiments

The isotopes of sodium and potassium used were ^{24}Na and ^{42}K supplied by the Atomic Energy Research Establishment, Harwell.

Before counting, the yeast samples were suspended in 5N-NaOH (1 ml./g. yeast). The activities of such suspensions were then measured by a Geiger-Müller tube (G.M. 4, Dynatron Radio Co.) the counts being registered on a Dynatron scaling unit, type Sc 200 (as supplied by the Dynatron Radio Co., Maidenhead). Corrections for decay were made from a curve or by using a control solution counted alternately with each sample.

Chemical determinations

Sodium and potassium. These were determined partly with the Barclay flame photometer and later with the Beckman flame photometer (Conway & Moore, 1954).

Bicarbonate and chloride ions. These were determined by a microdiffusion procedure (Conway, 1950*a*).

Experimental procedure

Preparation of the Na-*yeast.* Na-yeast was prepared by allowing washed baker's yeast to ferment for 2 hr. in 20 vol. of 0·2M sodium citrate plus 5% (w/v) glucose. After being washed twice in 20 vol. of tap water, the Na content of the sample lay, with few exceptions, within the range 50–80 m-moles Na/kg. centrifuged yeast. Concentrations are expressed throughout per kg. centrifuged yeast.

Determination of the 'active' sodium excretion. The freshly prepared Na-yeast was washed twice in 20 vol. of tap water and suspended 1:20 in the various solutions investigated. A stoppered bottle of about 500 ml. capacity was half filled with the yeast suspension and then placed in a shaker and samples were removed at intervals with a pipette. The samples were centrifuged at once and the centrifuged yeast was washed twice in about 20 vol. of tap water (which contained only 3 parts Na and 0·5 part K per million) before determining the content of Na etc. or radioactivity when isotopes were used. The centrifuged yeast sample was not washed when 0·1M-KCl or tap water was used as the suspending fluid, and the Na content only was required, as the Na in the fluid interspace between the cells is negligible. Such experiments were carried out at room temperature unless otherwise stated.

Occasionally also even when 20 vol. of a suspending fluid containing 0·1M-NaCl were used the centrifuged sample was not washed but an allowance made for the Na space of the yeast cell, into which Na and K ions freely diffuse, altogether a correction amounting to 33% by volume of the centrifuged yeast (Conway & Downey, 1950).

Experiments with inhibitors. Inhibitors such as cyanide or iodoacetic acid were introduced as their appropriate neutral salts in the suspending solution, to make 0·002M strength. With metal inhibitors (Hg, Pb, etc.) the suspending fluid consisted of 0·02M-NaCl plus 0·025M-KCl including small measured amounts of the inhibitor used. After centrifuging a sample of the suspension, the centrifuged yeast was washed twice with 0·1M-NaCl, and allowance made in the subsequent analyses for the passive Na entrance into the 33% region as considered above.

Determination of oxygen uptake. This was carried out with conventional manometric technique using 1 or 2 ml. of the Na-yeast suspension (1 in 20 usually) with 0·2 ml. M-KOH (with small strip of filter paper dipping in) in the central chamber for absorbing the CO_2 and shaking at 120 cyc./min. Readings were begun after 15 min. equilibration. Simultaneous determinations of the Na excretion from the yeast were made, the suspensions being shaken in a bath at the same temperature.

RESULTS

Demonstration of the active extrusion of sodium ions from yeast

Portions of twice-washed Na-yeast were suspended in 20 vol. of the following fluids: (*a*) 0·1M-NaCl, (*b*) tap water, (*c*) 0·1M-NaCl + 0·1M-KCl, (*d*) 0·1M-KCl. After varying times, samples of the suspensions were removed, centrifuged, and the yeast was analysed for sodium.

The Na content of the yeast at zero time was approximately 60 m-moles/kg. of centrifuged yeast. In Fig. 1 it will be seen that there was no net excretion of Na ions from the Na-yeast immersed in 0·1M-NaCl in 20 hr. There was, however, a net excretion into water, which came practically to an end after 3–4 hr. When 0·1M-KCl was present in the suspending fluid as well as 0·1M-NaCl there was a rapid excretion of Na ions, the rate differing little if any from that when 0·1M-KCl was present alone.

Ions exchanging with or accompanying the excreted sodium ions

When the Na ions were excreted into a medium containing 0·1M-KCl, the K ions taken up by the yeast were very similar in quantity to the Na ions excreted (Table 1). There was practically no loss of

K from the Na-yeast during the excretion of Na ions into tap water. In fact there was a slight increase after 24 hr., but this was probably within the total sampling error.

When the Na excretion occurs into water it must be either in exchange for H ions or accompanied by HCO_3 ions or by organic acid anions. Exchanges with H ions did not occur to any appreciable extent, as the pH of the originally unbuffered suspension remained on the average at about 6·2.

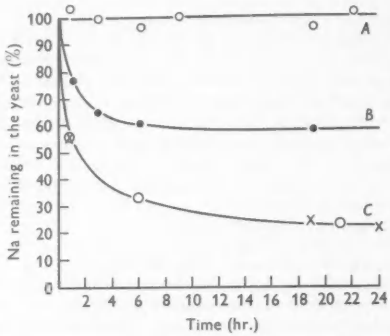

Fig. 1. The curves show the excretion rate of Na at room temperature into 20 vol. of various suspending fluid. Curve *A*, 0·1 M-NaCl; curve *B*, tap water; curve *C*, 0·1 M-NaCl plus 0·1 M-KCl (crosses) and 0·1 M-KCl (circles). The yeast contained approximately 60 m-moles/l. at zero time.

Table 1. *Changes of* Na *and* K *content of* Na*-yeast preparations on suspending in* 0·1 M-KCl *and in water*

At zero time Na^+ content = 64·9 m-moles/kg. yeast, K^+ content = 84·7 m-moles/kg. yeast.

Time of suspension (hr.)	Change in content (m-moles/kg. centrifuged yeast)			
	In 0·1 M-KCl		In water	
	Na	K	Na	K
0·5	−20·6	+25·3	− 7·9	−0·3
1	−36·8	+39·8	−14·0	−0·8
3·25	−40·9	+41·2	−19·8	+1·0
19	−47·8	+41·8	−23·4	+3·7

Estimations of the total CO_2 of the suspending fluid were made just after suspending and for different Na-yeasts after 60, 90 and 120 min. The increase of HCO_3 ions was calculated from the pH value and the total carbon dioxide and is shown in Table 2. Organic acid anions are considered to make up the remaining balance and their excretion in approximately such amount was confirmed by a study of electrometric titration curves. It is possible that the bicarbonate appearing in the suspending fluid was only indirectly formed following small exchanges of Na for H ions and restoration of the pH value by carbon dioxide but a considerable amount of bicarbonate was present within the cells of the freshly prepared and washed Na-yeasts. Thus there was a total acid-labile carbon dioxide content of 27·2 m-moles/kg. in the Na-yeast at zero time and only 15·0 m-moles/kg. centrifuged yeast after 2 hr. suspension. The Na content of this yeast changed from 55·4 to 47·3 m-moles/kg. over this period.

The nature of the organic acid anions was not determined but they were probably mostly succinate ions. Succinic acid is freely excreted by fermenting yeast (Conway & Brady, 1947).

The specificity of the sodium-excreting mechanism

Na-yeasts containing ^{42}K were prepared by fermenting for a short time in ^{42}K-labelled 0·1 M-KCl containing some glucose, a companion sample being treated in unlabelled KCl. The samples were then washed in water, centrifuged and Na-yeast prepared from them in the usual manner.

On suspending samples of each of these yeasts in unlabelled and labelled 0·1 M-KCl and measuring the counts of the centrifuged and washed yeast it was found that in 3 hr. there was practically no movement of K ions out of the cell (2–3 m-moles/kg.) but there was a considerable movement (approximately 45 m-moles) into the cell (Table 3). Table 4 shows that when Na-yeast was excreting Na ions into 0·1 M-NaCl plus 0·1 M-KCl, the external Na being labelled, there was practically no entrance of labelled Na, i.e. the movement of the Na ions was in the opposite direction to the K ions.

Table 2. *Anions accompanying the* Na *excretion from* Na*-yeast into tap water*

All Na, K, HCO_3 and organic anion values are given as m-equiv./kg. centrifuged yeast.

Time (min.)	Na content of yeast at zero time	Na excretion	pH of suspending fluid	Increase	
				HCO_3^-	Organic anions
60	49	9·2	6·2	3·1	6·1
90	45	9·8	6·4	7·9	1·9
120	60	13·6	6·1	4·1	9·5

Table 3. *Passage of labelled* K *ions to and from the cells of a* Na*-yeast suspended in* 0·1 M-KCl *at room temperature*

The counts of the yeast and the external fluid were made under similar conditions as described in Methods.

	Relative counts		
		Na-yeast with unlabelled K, labelled external K	
Time after suspending (min.)	Na-yeast with labelled K, unlabelled external K. Counts after 5 min. taken as 100	Counts as percentages of external counts for similar vols.	Percentage ratio of specific counts for yeast K to that for the external fluid
5	100	10·5	13
180	97	45·0	38

Table 4. *Passage of labelled* Na *into a* Na*-yeast immersed in a solution containing* 0·1 M-NaCl *plus* 0·1 M-KCl, *the external* Na *ions being labelled*

Time (min.)	Na counts in yeast as percentage of external counts under similar conditions of counting	Na content of yeast as determined by the flame photometer (m-moles/kg. centrifuged yeast)
0	0·8	55·2
60	0·8	32·1
150	0·7	17·3

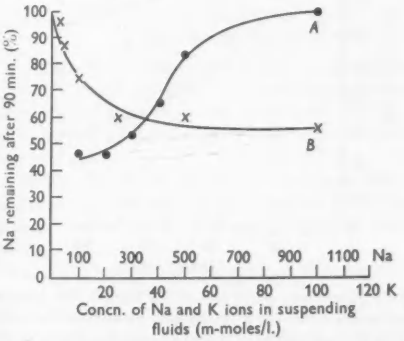
Na remaining after 90 min. (%)
A
B
100 300 500 700 900 1100 Na
20 40 60 80 100 120 K
Concn. of Na and K ions in suspending fluids (m-moles/l.)

Fig. 2. Percentage Na excretion from a Na-yeast after 90 min. at room temperature (1 in 20 suspensions). Curve *A* (dots), excretion into 0·1 M-KCl with varying amounts of NaCl; curve *B* (crosses), excretion into 0·1 M-NaCl with varying amounts of KCl.

The effect on the sodium excretion of varying the sodium and potassium content of the suspending fluid

The effect on the active excretion of varying the K and Na ion concentrations in the suspending fluid was studied in a series of experiments. It will be seen from Fig. 2 that when the suspending fluid was 0·1 M-NaCl there was no net excretion of Na ions, but very small additions of KCl caused a marked excretion. When the external KCl concentration was 25 m-moles/l., the effect of K was maximal.

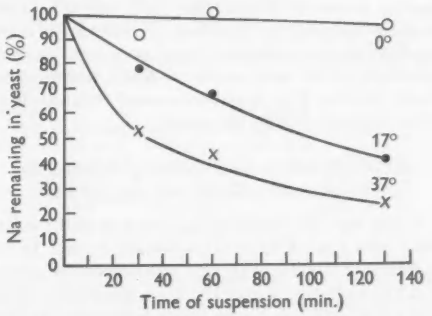

Fig. 3. Percentage excretion from a Na-yeast at 0, 17 and 37°. The suspending medium contained 0·1 M-KCl (1 in 20 suspensions).

When the external K concentration was maintained at 100 m-moles/l. and the Na concentration varied, the level of Na concentration had to reach about 420 m-moles/l. to reduce the excretion to one-half the maximum value (Fig. 2).

Effect of temperature and of pH *on the excretion of sodium from sodium-yeast*

The effect of temperature on the Na excretion into 0·1 M-KCl was studied (only to a small extent) by comparing the excretion rates at 0, 17 and 37° (Fig. 3). The excretion rate was very slow at 0° and a scarcely appreciable quantity of sodium was lost. More detailed data would be required for the determination of the activation energies for the overall process which are likely to differ over various temperature ranges; also owing to the complexity of the process involved they would be unlikely to provide useful information.

The effect of varying the pH of the external fluid between 4·0 and 7·0 was investigated. The suspending fluid was 0·1 M-KCl + 0·1 M-NaCl and the pH was varied by including different amounts of HCl on the acid side and of $NaHCO_3$ when studying pH effects a little above the neutral level. It was

decided to use HCl and $NaHCO_3$ to avoid any possible complications from organic buffering. It was found that practically no effect was produced by a change of pH from 4·0 to 6·0, but at 7·0 a fall of about 15% occurred.

Effect of azide on excretion of sodium into 0·1 M sodium chloride. Though Na-yeast suspended in 0·1 M-NaCl did not excrete any net quantity of Na ions, considerable exchange could be demonstrated if the Na-yeast was suspended in labelled NaCl (Table 5). The efflux and influx must be equivalent and from the efflux data, half of the Na ions originally in the yeast had crossed the membrane in 116 min. Sodium azide (0·002 M) reduced this efflux rate to about one-half, half the Na ions now passing across in about 260 min., but it almost abolished the influx. The effect of azide under these conditions was, therefore, to cause a net outward excretion of Na ions into 0·1 M-NaCl, which up to about the first 2 hr. was of the same magnitude as the net excretion into tap water.

Effect of azide on the excretion of sodium into 0·1 M potassium chloride and into tap water

When the Na-yeast was suspended in 0·1 M-KCl there was a rapid excretion of sodium ions. In the presence of 0·002 M azide this was decreased but the residual excretion was of the same magnitude as the excretion into tap water, or as the excretion into 0·1 M-NaCl with azide (Fig. 4). Fig. 4 also shows that the excretion into tap water was not affected by azide.

Table 5. *Exchange of labelled* Na *between* Na-*yeast and* 0·1 M-NaCl

In studying the entrance of Na into the Na-yeast, only the external Na was labelled, and for the excretion of Na only the internal Na was labelled.

The figures for the entrance rate are obtained from the expression $\frac{100 \times (\text{counts per g. yeast})}{\text{counts per ml. exit fluid}} - 33$.

The figure 33 represents the interspace value plus that of the outer region into which the Na ions can diffuse freely (Conway & Downey, 1950) and 100 represents the m.-equiv. Na ions externally.

The figures for the excretion rate are obtained from the expression $-\frac{60 \times (\text{counts per g. yeast})}{\text{counts per g. yeast at zero time}}$. Here 60 represents the Na concentration as m.-equiv./kg. centrifuged yeast at zero time.

	Without azide		With azide (0·002 M)	
Time (min.)	Entrance of labelled Na	Excretion of labelled Na	Entrance of labelled Na	Excretion of labelled Na
60	—	19·8	0·6	13·8
120	—	30·6	2·3	20·4
180	36·7	37·8	3·5	25·2
210	39·2	39·6	4·3	26·2

Under all conditions studied azide blocked the active uptake of K and Na ions. This inhibition of K ion uptake during fermentation has been shown in a previous communication (Conway, Brady & Carton, 1950). The effect on the influx of K ions into ordinary resting yeast suspended in 0·1 M-KCl and shaken with air or oxygen is shown in a later section of the present paper. The following experiments illustrate this azide effect with Na-yeast.

Effect of azide (0·002 M) *on the uptake of potassium by a sodium-yeast suspended in* 0·1 M *potassium chloride*

These experiments were carried out as for the previous group, the K content of the yeast with and without azide being determined after different immersion times. The results are shown in Table 6. It will be seen that after 60 min. immersion the net uptake of K has been reduced from 27·0 to 3·4 m-moles/kg. or nearly eliminated; between 20 and 60 min. no measurable uptake of K occurred.

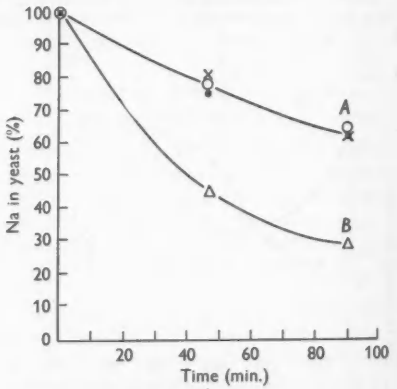

Fig. 4. Percentage excretion of Na from a Na-yeast into tap water and 0·1 M-KCl with and without azide (0·002 M) (1 in 20 suspensions). Curve *A*, into tap water (circles); into tap water plus azide (0·002 M) (dots) and into 0·1 M-KCl plus azide (0·002 M) (crosses); curve *B*, into 0·1 M-KCl.

Table 6. *Effect of azide* (0·002 M) *on the* K *uptake and* Na *excretion of* Na-*yeast*

Na and K values in yeast are given as m-equiv./kg. of the centrifuged material. Samples of Na-yeast were suspended (1 in 20) in the various fluids at 18°.

	Without azide		With azide	
Time of suspension (min.)	Na content	K content	Na content	K content
0	59·4	97·0	59·4	97·2
20	48·8	105·4	49·0	100·6
40	42·2	117·0	47·0	100·0
60	32·0	124·0	43·0	100·6

Table 7. *Effect of* 2:4-*dinitrophenol* (0·002 M) *on the* Na *excretion and* K *uptake of* Na-*yeast*

Na and K values in yeast are given as m-equiv./kg. of the centrifuged material. Samples of Na-yeast were suspended (1 part in 20) in the various fluids at 18°.

Time of suspension (min.)	NaCl (0·002 M) in tap water	2:4-Dinitrophenol (0·002 M) in tap water	KCl (0·1 M)		2:4-Dinitrophenol (0·002 M in 0·1 M-KCl)	
	Na content	Na content	Na content	K content	Na content	K content
0	54·4	54·4	54·2	68·5	54·2	68·5
30	45·3	44·4	37·4	87·0	37·2	71·8
60	42·2	41·2	24·4	92·2	30·0	76·4
90	39·5	39·6	15·8	102·0	28·3	76·4

Table 8. *The effect of cyanide and anoxia on the* Na *and* K *content of yeast suspended in tap water and in* 0·1 M-KCl

The washed Na-yeast was suspended in 20 times its volume of fluid at 18° for 90 min. Na-yeast immediately after suspending had 62 m-moles Na^+/kg. and 78 m-moles K^+/kg. of centrifuged yeast.

Conditions	Na changes (m-moles/kg. centrifuged yeast)		K changes (m-moles/kg. centrifuged yeast)	
	In tap water	In 0·1 M-KCl	In tap water	In 0·1 M-KCl
No inhibitor, oxygen present	−13·7	−38·0	−0·8	+33·3
No inhibitor, oxygen absent	−3·8	−8·6	−1·7	−0·3
Cyanide (0·002 M) and oxygen present	−5·0	−13·6	−0·0	−1·1

Effect of 2:4-*dinitrophenol on the excretion of sodium ions*

Samples of Na-yeast were suspended in tap water (1:20) containing 0·002 M 2:4-dinitrophenol, the pH of which had been adjusted to 6·0 with NaOH, the suspending fluid in the control mixture containing 0·002 M-NaCl.

The results shown in Table 7 indicate that like 0·002 M azide, 2:4-dinitrophenol had no effect on the Na excretion when no KCl was present in the suspending fluid.

When 0·1 M-KCl was present in the suspending fluid and the excretion of Na ions in such a suspension, without inhibitor, was much increased thereby, then the presence of 0·002 M 2:4-dinitrophenol caused a marked inhibition. This was not so marked as with 0·002 M azide, the Na excretion not being reduced to the level of that into tap water; this might be expected to occur with a higher dinitrophenol concentration.

Effect of cyanide and oxygen lack on the excretion of sodium ions from sodium-yeast

The inhibition produced by cyanide and anoxia is shown in Table 8. Whether the Na excretion was occurring into tap water or into 0·1 M-KCl, cyanide (0·002 M) and anoxia inhibited the excretion very largely. The uptake of K was completely inhibited by cyanide and by anoxia.

The active transport of Na ions in non-fermenting yeasts is thus very largely, if not entirely, dependent on electron transference to oxygen. It may be noted here that where the effect of oxygen lack was being studied complete absence of oxygen from the yeast suspensions was probably rare.

The effect of cyanide and azide on the potassium-carrier in ordinary resting yeast

Conway & Moore (1952) showed that the influx of labelled K was extremely slow into gently stirred resting yeast. If the yeast was shaken with air or oxygen the entrance rate was very much more rapid, but this increased rate was inhibited by cyanide and azide. In the present experiments yeast samples from the yeast factory were washed, and suspended in 20 vol. 0·1 M-KCl labelled with ^{42}K, with and without sodium azide (0·002 M) or sodium cyanide (0·002 M). The suspensions were well shaken with air or oxygen throughout. Samples were removed at intervals for estimations of ^{42}K in the yeast. The samples were centrifuged and counts were made in each case on the centrifuged yeast suspended in an equal volume of water. Fig. 5 shows the effect of the inhibitors. The points give the counts observed minus the count at 5 min. They represent, therefore, the entrance of ^{42}K into the yeast cells (from 5 min. onward) apart from the interspaces and the outer cell region into which K and Cl ions diffuse freely (Conway & Downey, 1950). It is obvious that an active carriage of K ions was

proceeding in the resting yeast which was shaken with air or oxygen, and that this was inhibited by azide and cyanide.

The effect of metallic ions on the active excretion of sodium from sodium-yeast

Sodium-yeast was suspended in a solution containing 0·1 M-NaCl, 0·025 M-KCl and various concentrations of the metallic salt. A control in which no metallic ions were included was set up at the same time, and the suspensions shaken for 60–90 min. Samples were then centrifuged and the yeast was washed twice quickly with 20 vol. of 0·1 M-NaCl, allowance being made for interspace and outer region (altogether 33 %) in calculating the Na inside the yeast membrane. The results are summarized in Table 9.

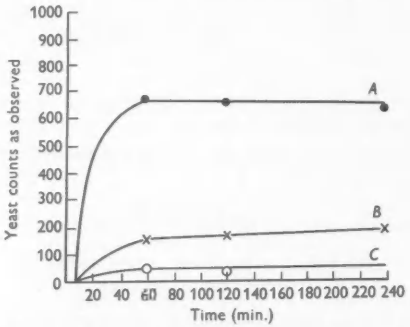

Fig. 5. Relative rates of entrance of K into untreated yeast (1 in 20 suspensions) in labelled 0·1 M-KCl with and without inhibitors. Curve *A*, without inhibitors; curve *B*, with azide (0·002 M) in the suspending fluid; curve *C*, with cyanide. The points give the counts for the yeast, after subtraction of the 5 min. values.

Several of the heavy metals, such as uranium, mercury, gold and silver, are powerful inhibitors of the process of Na secretion and are effective in one to a few parts per million. Other heavy metals such as lead, platinum and iron have comparatively little effect. When the results obtained are studied in relation to the number of electrons in the two outermost orbits of the metals, an interesting relation appears, which may be expressed in the following statements:

(*a*) A metal will cause marked inhibition if the second outermost orbit contains 18 electrons and the outermost contains not more than two electrons.

(*b*) The inhibition produced by the metals in group *a* increases with the magnitude of the atomic number.

Uranium holds an exceptional position in so far as it is among the most powerful inhibitors but does not come into group *a*. At the same time it may be noted that uranium was added as uranyl acetate, so that the uranyl ion and not the uranium ion was present in the mixture.

It will be seen from Table 9 that when the reciprocal number of the concentration (expressed as

Table 9. *Inhibition of the* Na *excretion (in exchange for* K*) by heavy metals*

	Atomic number	Concentration causing 50 % inhibition (m-moles/litre)	Reciprocals of such milli-molarities	Electrons in outer orbits
Metals with two outermost orbits containing 18 and 0–2 electrons				
Hg	80	$0{\cdot}74 \times 10^{-2}$	135	18, 2
Au	79	$0{\cdot}91 \times 10^{-2}$	110	18, 1
Ag	47	$1{\cdot}98 \times 10^{-2}$	50	18, 1
Pd	46	$2{\cdot}75 \times 10^{-2}$	36	18, 0
Cu	29	$15{\cdot}0 \times 10^{-2}$	6·7	18, 1
Zn	30	$107{\cdot}1 \times 10^{-2}$	0·9	18, 2
Metals with the two outer orbits containing 18 and 4 electrons				
Pb	82	410×10^{-2}	0·24	18, 4
Metals with second outermost orbits containing less than 18 electrons				
Pt	78	$71{\cdot}7 \times 10^{-2}$	1·4	17, 1
Ba	56	>100	<0·01	8, 2
Ni	28	>10	<0·1	16, 2
Co	27	>10	<0·1	15, 2
Fe	26	1·52	0·7	14, 2
Mn	25	>10	<0·1	13, 2
Cr	24	>4	<0·25	13, 1
Ca	20	>100	<0·01	8, 2
Al	13	>1	<1·0	10, 3
Mg	12	>100	<0·01	8, 2

Uranium with an atomic number of 92 is an apparent exception to the above grouping, as its two outermost orbits contain 12 and 2 electrons and the molarity for 50 % inhibition was found to be $0{\cdot}92 \times 10^{-5}$. The uranium, however, was added as the uranyl ion as contained in uranyl acetate. Sn showed no inhibition in the strengths used, but for solubility reasons the value could not be determined.

m-moles/l.) producing 50% inhibition exceeds 2·0, the metal has 18 electrons in its second outermost electron ring and not more than 2 electrons in its outermost. Palladium is listed for convenience as having no electron in the outer ring. Lead with 18 and 4 electrons in the outer rings has very little inhibiting action; the reciprocal number being 0·24. Zinc with 18 and 2 electrons in the outer rings and a reciprocal number of 0·9 has a smaller effect than would be anticipated. Yet this value is higher than that of the other metals of similar atomic number. (The reciprocal number of aluminium with atomic number of 13 is less than 1·0.) Platinum with the very high atomic number of 78 has a reciprocal number of only 1·4, only slightly exceeding that of zinc.

It may be concluded that the two rules given above are well borne out by the results, the most notable exception being uranium.

The relation of the oxygen consumed to sodium ions actively excreted from the sodium yeast suspended in 0·1 M *potassium chloride*

The present experiments, though adequate for their purpose in demonstrating a significant relation between oxygen utilization and Na excretion, may be considered as preliminary studies in this field.

Sodium-yeast was suspended 1 in 20 in 0·1 M-KCl; 1·0 ml. was immediately introduced into the Warburg apparatus for measurement of the oxygen uptake at room temperature; a larger sample of the suspension was placed in a flask and shaken well in a bath at room temperature for measurement of the Na excretion. The ratio of m-moles Na ions excreted to m-moles oxygen taken up was calculated for the period 20–80 min. after making the suspension.

The early experiments appeared to indicate that at least near the beginning of the Na excretion there was a limiting value of 4·0. On much further experimentation it was found that the oxygen consumption of the Na-yeast depended to a surprising extent on the previous history of the yeast, and could vary by as much as 5–10 times. With the higher oxygen consumption levels the ratio was much reduced at all periods of the excretion. For this reason we are now not inclined to attach much significance to the earlier results.

DISCUSSION

The various facts given in the Results section are well interpreted by the 'redox pump' theory, which is chiefly concerned with the manner in which the energy released in electron transference may be directly used for the performance of osmotic work (Conway, 1951, 1953).

In such a theory the carriers of the cations are considered to be respiratory enzymes in the reduced state. When oxidized the carriers release the ions, transferring electrons simultaneously to systems of higher redox potential. The nature of the energy transfer in performing osmotic work of such a kind has already been discussed (Conway, 1951, 1953). In the process by which the Na ions are excreted the carrier receives either H atoms or electrons, then attaches Na ions, and presents the Na-carrying group to another system, the electrons being transferred thereto and the Na ions released. This second system now picks up K ions and the process is reversed, the K ions being introduced into the cells and electrons transferred to a system with higher redox potential and ultimately to oxygen. If no K^+ is present outside the cells, then Na^+ is excreted at a slower rate with accompanying anions, the slower rate arising from the greater difficulty of bringing out anions than bringing K^+ into the cell.

Evidence for two carriers

The existence of two different carriers or carrier processes, one bringing out Na^+ and the other carrying in K^+, is strongly supported by the evidence, apart from the question of the carriers being redox systems. Such evidence turns on the specificity of carriage of Na^+ outward and of K^+ inward, and also on the different action of some inhibitors. Thus Na^+ is excreted accompanied by anions, when Na^+ in yeast is suspended in tap water (Table 2). At the same time no appreciable amount of K^+ is excreted, though the K^+ content of the yeast cell may be greater than the Na^+ content (Table 1); also, using labelled ions, it has been shown that when Na^+ is being excreted in exchange for K^+ there is no appreciable efflux of K^+ (Table 3). The Na^+ carrier is thus specific for Na^+ to a high degree compared with K^+. The reverse is true for the active uptake of K^+ into a Na^+ yeast, since Na^+ must be present in about 20 times the concentration of K^+ before it is taken up at an equal rate (Fig. 2, Table 4).

From this the conclusion may be drawn that there are two separate carriers or carrier-processes, one of which may be termed for convenience the Na^+ carrier and the other the K^+ carrier (though it is to be understood that when the external Na^+ content greatly exceeds the K^+ value, it will be taken in freely on the K^+ carrier).

This conclusion is strongly supported by the effect of inhibitors. When the Na-yeast is excreting Na^+ into tap water, azide (0·002 M) has no effect on the rate of Na^+ excretion (Fig. 4). If the Na-yeast excretes Na^+ into 0·1 M-KCl, it does so at about 2–3 times the rate into tap water (Fig. 1) the Na^+ exchanging for K^+ (Table 1). With such conditions azide (0·002 M) has a considerable inhibiting effect

on the Na^+ excretion. But the fact that by isotopic studies it appears that the uptake of K^+ is practically completely inhibited, and that the Na^+ excretion returns to the same level as excretion into tap water, shows that the inhibition has been exercised on the K^+ carrier.

In this connexion it is of much interest to consider the condition where the Na-yeast is suspended in 0·1 M sodium chloride. While no net excretion of Na^+ occurs over many hours, from isotopic studies it is shown that Na^+ is being freely transported in both directions. If azide (0·002 M) is included, the influx of Na^+ is almost entirely inhibited, and there results a net excretion of Na^+ similar in amount to the excretion into tap water (Table 5 and Figs. 1 and 4). Here it would appear that without any K^+ outside, the Na^+ is brought in on the K^+ carrier which is inhibited by azide and now Na^+ is excreted (accompanied by anions) as if into tap water or into 0·1 M potassium chloride with azide included (Fig. 4).

Evidence that the carriers are respiratory enzymes rather than substances using phosphate bond energy directly for the transport energy

(*a*) The excretion of Na ions from a Na-yeast whether K ions are present or not outside is inhibited by cyanide (0·002 M) or by anoxia (Table 7).

(*b*) After the uptake and release of Na ions, the succeeding uptake and release of K ions by another and quite distinct system is not well accounted for by phosphate bond energy but is very well interpreted by electron transference across redox systems.

(*c*) Azide (0·002 M) has no effect on the specific Na ion carrier, but azide abolishes the new formation of adenosine triphosphate or of organically bound phosphate (Spiegelman, Kamen & Sussman, 1948; Conway & McGovern, unpublished observations). Similarly, 2:4-dinitrophenol (0·002 M) does not affect the Na carrier, or the Na excretion into tap water, and it is generally considered to uncouple phosphorylation from respiration without inhibiting the latter.

The fact that 2:4-dinitrophenol (0·002 M) inhibits the K carrier does not necessarily imply that this carrier uses phosphate bond energy directly. Phosphorylation may be required at some stage of the electron transference in which the K carrier is involved.

(*d*) Calcium or magnesium ions even at a concentration of 0·1 M have no effect on the active excretion of Na^+ and the uptake of K^+. But Ca^{2+} and Mg^{2+} would displace Na^+ and K^+ from such polyphosphate carriers *in vitro*.

Connected with this, it has recently been shown here (Conway & Beary, unpublished observations) that Mg^{2+} is steadily taken up by yeast suspended in 20 times its volume of a solution containing 0·05 M magnesium chloride + 5 % (w/v) glucose and stirred by bubbling with oxygen, the mixture being maintained at a pH of 6·2 by small additions of 0·1 N sodium hydroxide. The uptake of Mg^{2+} is inhibited by K^+ in the external fluid. Practically complete inhibition is obtained by a K^+ concentration as low as 0·01 M potassium.

(*e*) That the K carrier is of a redox kind is shown also by the following experiment. Redox dyes in small concentration (M/10 000) in the suspending fluid (1:20) with the yeast fermenting anaerobically can alter markedly the external redox potential as determined by a platinum electrode in the suspension (Conway, 1953). With dyes of different characteristic potentials the external potential can be considerably lowered or increased above the value without the dyes. Complete inhibition can be produced when the potential is lowered by about 60 mv, and corresponding stimulation can occur when the potential is raised. There is in fact a linear relation between the relative change of the K^+ transport and the redox potential. (Such experiments were described at the Bangor Congress on Active Transport, 1953, and it is hoped to describe them in more detail with Mr Kernan in a subsequent paper.) While the inhibition of the K carrier system by 2:4-dinitrophenol points to a phosphorylation requirement in the active transport this of course need be only indirect.

The evidence appears strongly in favour of respiratory ferment carriers and the direct utilization of electron energy as in the 'redox pump', though the question of the specificity of the carriers implies closer linkage than that given by long range electrostatic forces. The complex may be considered somewhat in the nature of the linkage of an enzyme with its substrate.

SUMMARY

1. The excretion of Na ions from a 'sodium-yeast' (containing about 60 m-moles/kg.) into 0·1 M-KCl is nearly 3 times faster than into water and there is a nearly quantitative exchange of K for Na ions. When Na ions are excreted into water they are accompanied by bicarbonate and organic acid anions and the excretion ceases when about half the Na has been excreted. There is no net excretion of Na ions into 0·1 M-NaCl.

2. When a sodium-yeast is suspended in 1:20 of 0·1 M-NaCl, no net excretion of Na^+ occurs over many hours. If suspensions are made with a series of solutions containing 0·1 M-NaCl and various concentrations of KCl, the excretion of Na^+ increases with the K^+ concentration of the suspending fluid, but this effect is maximal at a K^+ concentration of 0·04 M-KCl.

3. When Na^+ is excreted under similar conditions into 0·1 M-KCl with NaCl ranging from zero to 1·0 M, little reduction in the rate of Na^+ excretion occurs up to an external Na^+ value of 0·2 M. At an external value of 0·43 M the Na excretion is approximately 50 % that at zero Na^+ concentration.

4. The existence of two separate carriers for Na (outwards) and for K (inwards) is shown by the relative specificity of the transport in each case and the different action of inhibitors. Where Na ions (0·1 M) without any K ions are present in the suspending fluid Na ions can be carried inwards on what may be regarded as normally the K carrier.

5. The Na carrier excreting Na is inhibited by cyanide (0·002 M) and anoxia; it is not inhibited by azide (0·002 M) or 2:4-dinitrophenol (0·002 M).

6. The K carrier involved in the uptake of K^+ (or of Na^+ if no K^+ is present outside, and Na^+ is present in quantity) is inibited by anoxia, and by cyanide, azide and 2:4-dinitrophenol at concentration of 0·002 M.

7. The results are shown to support strongly the theory of a 'redox pump' for the biological performance of osmotic work.

8. Various metallic ions can greatly inhibit the active excretion of Na ions into 0·1 M-KCl. Inhibition depends on the magnitude of the atomic number and on the presence of 18 electrons in the second outermost orbit, and not more than two in the outermost.

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Studies of Sebum

5. THE COMPOSITION OF SOME SEBUM-LIKE MATERIALS OF HUMAN ORIGIN

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Apart from true sebum or surface-skin lipid there exist a number of materials which are alleged to originate from true or modified sebaceous glands. Some of these materials such as cerumen or ear wax, smegma and vernix caseosa are normal in origin while others, for instance dermoid cyst lipid, are pathological. The latter material is of interest. Dermoid cysts frequently grow to relatively enormous size in the ovaries; the cysts are hollow and are lined with what appears to be epidermal tissue. The cyst is usually filled with a mixture of water, fatty material and hair. The fatty material has long been thought to be sebum (Sotnitchewsky, 1880), and while it has been investigated by numerous workers, no direct comparison has been made between its composition and that of sebum.

In order to obtain information about the origin of these sebum-like materials, ovarian dermoid cyst lipid, ear wax, vernix caseosa and epidermal cyst lipid have been analysed and the results are compared with those obtained with sebum (MacKenna, Wheatley & Wormall, 1950, 1952). Preliminary reports of part of this work have already been published (Wheatley, 1951, 1953*a*).

EXPERIMENTAL

Materials

Fatty material was obtained from two ovarian dermoid cysts and was found to contain little water. It dissolved readily in $CHCl_3$, and the lipid which remained after filtering and evaporating this $CHCl_3$ solution to dryness was analysed. Ear wax was obtained from European patients of this hospital and contained much dirt and extraneous material. The lipid was extracted from both single samples and bulked specimens by refluxing the material with $CHCl_3$

and filtering off the extraneous matter. The vernix caseosa was obtained by gently scraping the skin of newly born babies with a glass slide. The epidermal cyst was excised from a patient attending this hospital. Both these materials contained appreciable amounts of water, they were first dehydrated by refluxing with acetone and then extracted with $CHCl_3$. The combined acetone and $CHCl_3$ extracts were then evaporated to dryness and the last traces of water removed by adding small amounts of acetone and carefully distilling this off. The residue was then dissolved in warm $CHCl_3$, the solution filtered and evaporated to dryness.

Analytical methods

All melting points recorded here are uncorrected; micro-analyses were performed by Weiler and Strauss, Oxford.

Chemical constants. Iodine numbers and acid numbers were determined on suitable samples by the methods described in earlier work (MacKenna *et al.* 1950; Hodgson-Jones & Wheatley, 1952). Cholesterol was determined by the method of Kelsey (1939), phosphorus by the method of Fiske & Subbarow (1925) after digestion with H_2SO_4 and HNO_3, and nitrogen by a micro-Kjeldahl method.

Fractionation. The lipids were fractionated into free fatty acids, combined fatty acids and unsaponifiable matter by the method used for sebum (MacKenna *et al.* 1950). A heavy interface precipitate was formed when alkali was added to the ethereal solution of both ear wax and vernix caseosa; this rendered separation difficult.

Chromatography of the unsaponifiable matter. The method described for sebum unsaponifiable matter (MacKenna *et al.* 1952) was used. In most cases a sample of 1 g. was fractionated on a 20 g. column of alumina (Brockmann, grades II–III); with larger quantities of unsaponifiable matter correspondingly bigger columns were used. An attempt was made to replace the $CHCl_3$–petroleum mixtures, used as eluents, by simple substances (benzene, ether, etc.), as recommended by Nicolaides & Rothman (1953), but it was found that in order to obtain good separation with these solvents very careful selection of the alumina was necessary. For example, with grade II alumina benzene failed to elute the wax-alcohol fraction, while with grade III alumina benzene eluted both the wax alcohols and cholesterol. It was therefore decided to retain the use of mixed solvents and to follow the development of the chromatogram by collection of 100–300 ml. portions of eluate.

With both ear wax and vernix caseosa there was an appreciable amount of material which was insoluble in light petroleum and caused blocking of the column during the early stages of the chromatography. It was found that this blocking could be prevented if the column was covered with a thin layer of sand (2–3 mm. thick) before loading.

Paper chromatography. Alumina-impregnated paper was prepared as described by Bush (1952) and stored over saturated $CaCl_2$ soln. (relative humidity about 30%). The chromatograms were run by the ascending method using either benzene–light petroleum (b.p. 80–100°), benzene–$CHCl_3$ mixtures (equal parts by volume) or pure $CHCl_3$. The chromatogram was then sprayed with $SbCl_3$ in $CHCl_3$ and heated in an oven at 90° for about 3 min. A blue spot showed the presence of sterol-like substances. The activity of the paper varied slightly with each batch and the R_F values given below refer to individual experiments only. The presence of a particular substance was established by running three parallel chromatograms of the unknown substance, the authentic compound and a mixture of both. Fuller details of the paper chromatography procedures will be given in a subsequent paper.

Identification of squalene. Squalene was isolated as the hexahydrochloride in the following manner. The hydrocarbon fraction obtained by chromatography of the unsaponifiable matter was dissolved in either acetone or ether. The solution was cooled to −5° and saturated with dry HCl gas. After the mixture had been allowed to stand for several hours, the precipitate was collected, washed thoroughly with light petroleum to remove adhering hydrocarbons and twice recrystallized from acetone. The crystals thus obtained were submitted for analysis; only when a correct Cl content was obtained for the hexahydrochloride thus prepared was the presence of squalene considered to be established. The squalene content of the various lipids was then estimated by the method described in the preceding paper of this series (Wheatley, 1953*b*).

RESULTS

The general composition of the lipids from these sebum-like materials is shown in Table 1; while in Table 2 the results of the chromatographic analysis of the unsaponifiable matter from these lipids is given. The most notable differences between these

Table 1. *General properties of some lipids thought to be related to sebum*

	Source of lipids						
		Ovarian dermoid cyst			Vernix caseosa		
	Sebum	(*a*)	(*b*)	Ear wax (6 specimens)	(*a*)	(*b*)	Epidermal cyst
M.p. (°)	35–37	34	31	33–36	31	32	—
Iodine no.	53–63	56	79	34–74	46	59	48
Acid no.	59–73	0·6	0·3	47–64	5*	15	29*
Total cholesterol (%)	2·7–5·9	3·6	6·8	12·1–17·1	19·7	20·6	17·4
Total N (%)	0·42–0·49	Nil	0·07	0·9–1·92	0·32	0·44	—
Lipid P (%)	Nil	Nil	Nil	0·01–0·041	0·025	0·029	0·093
Free fatty acids (%)	22–32	0·6	0·5	29·9–36·2	2·3	9·4	14·7
Combined fatty acids (%)	28–41	70·2	70·8	8·9–20·8	33·3	42·1	4·3
Unsaponifiable matter (%)	27–36	27·3	28·2	27·0–39·7	27·0	48·6	57·7
Squalene (%)	3·3–8·7	3·7	10·2	1·0–12·0	-	2·6	Nil

* Calculated results.

Table 2. *The composition of the unsaponifiable matter of the sebum-like material* (%)

Fractions	Source of lipids: Sebum	Ovarian dermoid cyst (*a*)	Ovarian dermoid cyst (*b*)	Ear wax (*a*)	Ear wax (*b*)	Vernix caseosa
I. Hydrocarbons	37–46	13·2	39·0	18·3	9·3	6·4
II. Wax alcohols	17–25	56·2	41·2	19·6	22·0	13·0
III. Cholesterol	14–19	11·8	14·8	33·8	30·0	40·5
IV. Sterol-like substances and other compounds	4–11	7·8	3·1	6·3	10·2	12·3
V. Unidentified	6–13	7·8	2·1	18·9	18·1	16·5

lipids and sebum are the following. Free fatty acids are absent from dermoid cyst lipids while they are present in ear wax and vernix caseosa. The cholesterol content of ear wax and vernix caseosa is much higher than sebum. The hydrocarbon fraction is larger in sebum than in any of the other materials examined. There is a higher proportion of waxes in dermoid cyst lipid than in sebum, ear wax or vernix caseosa.

Examination of unsaponifiable matter

The various fractions obtained in the chromatographic analysis were further investigated. The composite results of these studies are recorded; where differences were observed between different specimens these have been indicated. Owing to the small amount of material available only a cursory examination of the fractions from ear wax and vernix caseosa could be made, but with the dermoid cyst lipids a much more detailed investigation was possible.

Dermoid cyst

Fraction I. This was a colourless viscous liquid which was composed largely of squalene (specimen 1, 88%; specimen 2, 81%). Urea adduct formation (Zimmerscheid, Dinerstein, Weitkamp & Matschner, 1950) showed the presence of only about 1% *n*-paraffins, and recrystallization from ethanol of the residue after removal of squalene gave only a small amount of solid hydrocarbons.

Fraction II. The crude fraction was a grey-white wax (m.p. 49·5°, specimen 1; 51·4°, specimen 2) which on recrystallization from acetone gave a white wax m.p. 65·6° (specimen 1); 60·0° (specimen 2). When large batches (20 g.) of unsaponifiable matter were fractionated on a long column (250 g. alumina) the first specimen showed three principal fractions. (*a*) A brown gum (4·4% of total unsaponifiable matter), iodine number 102, acetyl number 18, which failed to form an insoluble urea adduct. This was probably an oxidation product of squalene. (*b*) A soft white wax (18·9% of total unsaponifiable matter) iodine number 40, which when subjected to urea adduct formation (Tiedt & Truter, 1952) was shown to be composed of 32% *n*-alcohols (m.p. 48·2°, iodine number 23) and liquid branched-chain alcohols (iodine number 47). (*c*) A hard white wax (33·3% of total unsaponifiable matter) iodine number 13, composed of 41% *n*-alcohols (m.p. 62·8°; iodine number 3) and solid branched-chain alcohols (m.p. 55°, iodine number 18). The second specimen gave two fractions: (*a*) a soft white wax composed of 38% *n*-alcohols (m.p. 53·8°, iodine number 30) and liquid branched-chain alcohols (iodine number 40) and (*b*) a hard white wax composed of 39% *n*-alcohols (m.p. 61·8°, iodine number 3) and solid branched-chain alcohols (m.p. 42·8°, iodine number 21).

Fraction III. This was composed of almost pure cholesterol which was characterized as the dibromide (m.p. 117–18°) and the acetate (m.p. 114°). Estimation by Schönheimer's (1930) method showed that it contained a small amount (2·1% specimen 1; 1·6% specimen 2) of saturated sterol (probably β-cholestanol). The crude fraction gave a doubtfully positive Rosenheim test with large amounts (20–30 mg.) of material.

Fraction IV. The crude fraction was a brown gum which gave positive Rosenheim and $SbCl_3$ reactions, it was not found possible to crystallize either the crude material or the material resulting from benzoylating the crude fraction.

Preliminary investigations of the fraction were conducted on the first specimen. Careful chromatography of the crude fraction on a long column (30 cm.) of alumina gave a white solid fraction which gave a very strongly positive $SbCl_3$ test, but neither this material nor that resulting from benzoylation could be induced to crystallize. The benzoate gave a blue colour with acetic acid and H_2SO_4 (Haslewood 1939). When a further batch of the crude fraction was treated with digitonin in 95% ethanol an insoluble digitonide was obtained. This digitonide on treatment with pyridine and ether yielded a brown gum, but neither the gum nor the material resulting from benzoylating the gum could be induced to crystallize, and chromatography of the benzoylated material on alumina failed to achieve a purification. It was subsequently shown that when a paper chromatogram of the principal fraction, obtained by this chromatography, was run, two blue spots

were obtained (R_F 0·86 and 0·75 with benzene–light petroleum), the latter corresponding to 7-α-hydroxycholesterol benzoate. It was also observed that the 'digitonin' recovered from the digitonide after treatment with pyridine and ether still gave a positive $SbCl_3$ test and contained a digitonide not decomposed by this treatment. The material not precipitated from the crude fraction by digitonin gave brown colorations with the Liebermann–Burchard, Rosenheim and $SbCl_3$ tests and when a paper chromatogram of the material was run (chloroform) no blue spot was observed.

The fractions obtained by chromatography of 20 g. batches of unsaponifiable matter (250 g. column) were examined in the following manner. With specimen 1, paper chromatograms were run (chloroform) on each portion of eluate; a blue spot was observed with some of these fractions. These fractions were pooled and the pooled material (3·3 % of the total unsaponifiable matter) which contained 19 % sterol (calculated as cholesterol) was treated with digitonin. The preliminary separation was omitted with the second specimen and the whole of the crude fraction was treated with digitonin. The digitonides from both specimens were treated with pyridine and ether, when a brown gum was obtained (0·3 % total unsaponifiable matter with specimen 1 and 0·06 % with specimen 2). This gum was benzoylated and when paper chromatograms were run on the benzoylated material, a single blue spot was obtained (R_F 0·90, benzene–light petroleum) which was not identical with 7-α-hydroxycholesterol benzoate (R_F 0·70). The fraction was not pure, as the paper chromatogram showed an intense brown streak at the origin. The digitonide was then refluxed with xylene for 6 hr. when complete decomposition was effected. A gum was recovered after removal of the xylene *in vacuo* (0·29 % total unsaponifiable matter in specimen 1 and 0·12 % in specimen 2). This gum was benzoylated and paper chromatograms were run on the benzoylated material. The two specimens gave different results. The first specimen gave four principal constituents, three gave blue spots (R_F 1·0, 0·85, 0·55 with benzene–chloroform) and one lilac spot (R_F 0·91) while the second specimen contained three constituents, one a blue spot (R_F 0·49) and two lilac spots (R_F 0·87 and 0·56); traces of other constituents were also observed.

The fraction therefore contained several sterols or sterol-like substances which form insoluble digitonides. The digitonide of one of these is decomposed by treatment with pyridine and ether, the sterol was not identical with 7-α-hydroxycholesterol but may be oxidized to this substance. The other sterols form digitonides which are decomposed only by boiling xylene. It has not been possible to isolate a crystalline substance from any fraction, possibly owing to the complex nature of the mixture.

Fraction V. A dirty white solid which on recrystallization from methanol gave a white solid m.p. 79° (specimen 1); 78° (specimen 2).

Ear wax

Fraction I. A white viscous liquid containing about 85 % squalene. After removal of the squalene the residue was crystallized from ethanol to give a small amount of solid hydrocarbon. *Fraction II.* A dirty white material which on recrystallization from acetone gave a white wax, m.p. 47°. *Fraction III.* Almost pure cholesterol, characterized as the acetate (m.p. 113°) and the dibromide (m.p. 117–18°). *Fraction IV.* A brown gum which gave positive Rosenheim and $SbCl_3$ tests. Paper chromatography of the crude fraction showed a blue spot corresponding to that obtained with the same fraction from sebum. *Fraction V.* Recrystallization of the crude fraction from methanol gave a white solid m.p. 78–81°.

Vernix caseosa

Fraction I. This consisted of practically pure squalene and a trace of solid hydrocarbons. *Fraction II.* On recrystallization of the crude fraction from acetone a white wax m.p. 58·8° was obtained. *Fraction III.* Practically pure cholesterol which was characterized as the acetate (m.p. 114°). *Fraction IV.* The crude fraction gave positive Rosenheim and $SbCl_3$ colorations but paper chromatography failed to reveal the presence of a blue spot corresponding to sebum. *Fraction V.* This resembled the corresponding fraction obtained from sebum.

Epidermal cyst

There was insufficient material to subject to full chromatographic analysis. The absence of squalene was established and only a negligible amount of hydrocarbon could be detected.

DISCUSSION

An examination of the composition of four lipid materials thought to be related to sebum showed striking similarity in most cases, though definite differences were also shown to exist. It is interesting that dermoid cyst lipid, ear wax and vernix caseosa all contain a smaller hydrocarbon fraction than sebum and this is composed largely of squalene. The corresponding fraction from sebum contains only about 50 % squalene, the remainder being largely *n*-paraffins. It has been established that dermoid cyst lipid contains very little of *n*-paraffins, and there is a suggestion that only a small amount may be present in ear wax and vernix caseosa. It is therefore possible that the *n*-paraffins found in

sebum do not originate from the sebaceous glands and may be contaminants.

The large amounts of material available from the two dermoid cysts have enabled the unsaponifiable matter from this lipid to be investigated in greater detail than has yet been possible with sebum. When large samples of unsaponifiable matter were fractionated on long columns of alumina the wax alcohols tended to separate, the shorter-chained and unsaturated members travelling more rapidly than the longer-chained saturated ones. Urea adduct formation showed that the mixed fraction contained about 38% *n*-alcohols (10% of which were unsaturated) and branched-chain alcohols (30% of which were unsaturated). Various *n*-alcohols have been claimed to be present in dermoid cyst lipid, namely tetradecanol (Dimter, 1941), hexadecanol (Ludwig, 1897), eicosanol (Ameseder, 1907) and docosanol (Dimter, 1941). Mass spectrometry of the wax alcohols from human-hair lipid (Nicolaides & Rothman, 1953) has shown that the straight-chain alcohols exist as a homologous series ranging from C_{18} to C_{26} with odd as well as even members present. It has not been possible to submit the fractions from dermoid cyst lipid to mass spectrometry but it is considered highly possible that a similar homologous series exists in this material; this would easily explain the confused picture of the nature of the wax alcohol obtained by earlier workers. There is also some indication that the fractions from the two specimens of cyst lipid were slightly different.

Careful examination of the cholesterol and the following fraction from dermoid cyst lipid has revealed the presence of several sterols or related substances. The presence of a small amount only of saturated sterol (β-cholestanol) has been established, though Behmel (1932) claimed that this substance was present 'in abundance'. An indication has been obtained for the first time with any sebaceous substance that 7-dehydrocholesterol or a similar substance may be present. Several sterols were found to be present in fraction IV, which formed insoluble digitonides. The digitonide of one of these was decomposed on being treated with pyridine and ether. This was not identical with 7-α-hydroxycholesterol, but in one batch of material that had been subjected to somewhat involved manipulation the unknown sterol was found to be accompanied by the latter substance. It could therefore be related to 7-α-hydroxycholesterol and yield this substance on oxidation. The digitonides of the other sterols required treatment with boiling xylene to decompose them, at least three components were present and these were not identical in the two specimens of lipid examined. It is possible that certain of the substances are oxidation products of sterols present in the original material and it is proposed to investigate the problem in greater detail when larger supplies of material become available.

Summarizing the results of the examination of these sebum-like materials, we may say that ear wax resembles sebum that has been oxidized and contaminated with extraneous cholesterol and other substances. Vernix caseosa gives the picture one would expect of the sebum of an immature animal, for it has been shown by Nicolaides & Rothman (1952) that in the hair lipids of children the cholesterol content is higher and the squalene content lower than that from adults. Furthermore, the material probably contains sterol-like substances of maternal origin. Both ear wax and vernix caseosa can be regarded as of sebaceous origin; on the other hand, the epidermal cyst lipid is different from sebum and it is unlikely that this substance originates from true or modified sebaceous glands.

The dermoid cyst lipid resembles sebum in many ways but there are several differences that are not easily explained. One such difference is the absence of free fatty acids. It has been shown by Nicolaides & Rothman (1953) that the free fatty acid content of human hair lipid increases on storage, the liberated acids being formed by hydrolysis of the triglycerides present, and it is possible that the free fatty acids of human sebum may originate in such a manner by bacterial or other hydrolysis. Such hydrolysis would not occur in dermoid cyst lipid; but neither should it occur in vernix caseosa which has been shown to contain some free fatty acids even though less were present than in sebum. The presence of a much larger proportion of true waxes in dermoid cyst lipid is equally difficult to explain. Furthermore, there is strong evidence both from this examination of two specimens of the lipid and from the investigations of other workers that different samples of this lipid material vary appreciably in composition. The most likely interpretation of all these observations is that the lipid in dermal cysts represents a 'sebum' produced by a pathological process.

SUMMARY

1. Certain substances thought to be related to sebum (ear wax, vernix caseosa, dermoid and epidermal cyst lipids) have been examined and their composition compared with that of sebum.

2. It is concluded that ear wax and vernix caseosa are related to sebum and originate from true or modified sebaceous glands, that epidermal cyst lipid is not related to sebum and that dermoid cyst lipid represents a 'sebum' produced by a pathological process.

3. The unsaponifiable matter from the contents of two dermoid cysts has been examined in detail. The presence of straight- and branched-chain

alcohols has been established. Some saturated sterol (probably β-cholestanol) is present and a number of other sterol or sterol-like substances which form insoluble digitonides have been found.

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The Vitamin D Content of Tissues of Rats given Ergocalciferol

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In previously reported balance experiments, in which rachitic rats were given a single massive dose of 1 mg. of ergocalciferol, it was found that only about 6 % was recovered in the body (Cruickshank, Kodicek & Armitage, 1953). About half of it was present in the liver, which therefore appeared to be the principal store of the absorbed vitamin. It was impracticable in that investigation to examine the distribution in all the individual organs and tissues but the possibility existed that some organs, though small in size and therefore contributing little to the total content, might nevertheless have a relatively high concentration. Subsequent work, already briefly reported (Cruickshank & Kodicek, 1952), showed that this was so and details of the experiments are now presented.

EXPERIMENTAL

The design of the experiments is shown in Table 1. The experimental procedure was that already described (Cruickshank *et al.* 1953). Groups of rachitic rats were given by mouth 1 mg. crystalline ergocalciferol/rat and the vitamin D content of various organs in the fresh state, 24 and 48 hr. after dosing, was determined biologically on rats by the radiographic method of Bourdillon, Bruce, Fischmann & Webster (1931).

The determination of vitamin D was carried out in three series of assays, in which about 200 rats were used. The ranges of doses, in mg. of fresh material, were as follows: muscle, 100–1000; skin, 200–1000; kidneys, lungs and spleen, 15–60; brain, 125–500; adrenals, 32. Since in the previous work the whole carcass from undosed rachitic rats contained little or no vitamin D (Cruickshank *et al.* 1953), it was considered unnecessary to assay the individual organs of undosed animals with the exception of the adrenals, which when assayed were found to contain no vitamin D.

Table 1. *Design of experiments*

Each rat received 1 mg. ergocalciferol

Expt.	No. of rats	Sex	Day of killing	Tissues investigated
1	5	3 M. 2 F.	1st	Muscle, skin, kidneys
2	5	3 M. 2 F.	2nd	Muscle, lungs, spleen, kidneys, adrenals
3	6	3 M. 3 F.	2nd	Muscle, skin, brain

RESULTS

Table 2 shows the distribution of vitamin D in various organs on the first and second days after the administration of ergocalciferol. For comparison the vitamin D contents of liver and intestinal tissue found in previous experiments (Cruickshank *et al.* 1953) have been included. Although it was found that the liver contained most of the absorbed vitamin, our present results indicate that there is an appreciable amount also in muscle and skin. Each of these two tissues showed an apparent increase between the first and second days which barely reached the 5% level of significance ($P = 0{\cdot}03$ for muscle and 0·05 for skin). The kidneys contained an amount of the same order despite their small size. The lungs, spleen, brain and adrenals contributed little to the vitamin content of the body.

The kidneys contained a relatively high concentration of the vitamin. Thus on the second day the estimated value was 204 i.u./g., i.e. slightly though not quite significantly higher ($P = 0{\cdot}06$) than that found in the liver, 123 i.u./g. In lungs, spleen and adrenals the concentration was of the order of 100 i.u./g., while in muscle and skin it was relatively low and in brain negligible.

In Table 3 the sum of the vitamin contents in the individual tissues examined in the present studies (as given in Table 2) is compared with the total given by Cruickshank *et al.* (1953) for the 'carcass' (i.e. the total body minus the liver and intestines). For the rats killed on the first day after dosing, the total content of the muscle, skin and kidneys was estimated to be 620 i.u./rat, a value not significantly different from the previous estimate for the 'carcass' of 690 i.u./rat. For the rats killed on the second day, the total content of muscle, skin, kidneys, lungs, spleen, brain and adrenals was estimated to be 890 i.u./rat; this value is significantly higher ($P = 0{\cdot}04$) than the previous estimate, for the 'carcass', of 630 i.u./rat.

Statistical treatment

The statistical analysis follows closely that described in more detail in Part II of Cruickshank *et al.* (1953). The main features of the three series of assays are shown in Table 4.

Table 2. *Vitamin D content per g. fresh weight and per whole tissue of rats given* 1 *mg. ergocalciferol* (40000 *i.u.*)

In all tables the potency per g., when less than 100 i.u., was calculated to the first decimal, otherwise to the nearest whole number. The total content in i.u., when less than 50 i.u., was rounded off to the nearest unit; otherwise to the nearest 10, except for replicate determinations which are later averaged. Data for liver and intestinal tissue from Cruickshank *et al.* (1953).

	1st day. Vitamin D					2nd day. Vitamin D				
Tissue	Expt.	Mean wt./rat (g.)	(i.u./g. fresh wt.)	Total (i.u./rat)	95% fiducial limits (%)	Expt.	Mean wt./rat (g.)	(i.u./g. fresh wt.)	Total (i.u./rat)	95% fiducial limits (%)
Liver	—	3·74	385	1440	69–144	—	4·16	123	510	67–150
Intestinal tissue	—	4·66	53·7	250	69–145	—	4·70	19·2	90	68–147
Muscle	1	13·6	18·1	250	70–142	2	13·2	34·0	449	390* 78–129
						3	14·0	23·4	328	
Skin	1	10·5	14·5	150	72–139	3	10·5	22·4	240	73–136
Kidneys	1	0·82	269	220	72–138	2	0·89	204	180	70–143
Lungs	—	—	—	—	—	2	0·40	122	49	69–145
Spleen	—	—	—	—	—	2	0·19	99·8	19	66–152
Brain	—	—	—	—	—	3	1·44	6·9	10	68–147
Adrenals	—	—	—	—	—	2	0·032	104	3	48–208
Total (excluding liver and intestinal tissue)	—	—	—	620	—	—	—	—	890	—

* Arithmetic means of values for Expts. 2 and 3.

Table 3. *Comparison of results obtained from individual organs and from 'carcass' of rats given* 1 *mg. ergocalciferol* (40000 *i.u.*)

	1st day		2nd day	
		95% fiducial limits (%)		95% fiducial limits (%)
Total i.u. from individual organs (Table 2)	620	82–122	890	85–117
Total i.u. in 'carcass' (Cruickshank *et al.* 1953)	690	67–150	630	75–133

Table 4. *Summary of vitamin D assays for Expts.* 1–3

Expt.	Common slope ±S.E.	Residual mean square (with degrees of freedom)	Variance ratios and significance	
			Between slopes	Departure from linearity
1	3·79±0·39	0·6374 (59)	1·70 ($0·10<P<0·20$)	<1 ($P>0·20$)
2	4·23±0·42	0·9335 (74)	1·55 ($0·10<P<0·20$)	<1 ($P>0·20$)
3	3·85±0·35	0·5263 (58)	<1 ($P>0·20$)	2·30 ($0·05<P<0·10$)

The estimate of the vitamin content in the muscle on the second day (for which two replicate groups were available) was obtained by calculating the arithmetic mean of the two separate estimates (Table 2). The fiducial limits for this pooled estimate and for the totals shown in Table 3 were obtained by applying the formula given at the end of Part II of Cruickshank *et al.* (1953).

The significance tests for differences in vitamin content between different tissues, or for the same tissue on different days, are performed by obtaining the difference between the logarithms of the two values, and calculating its standard error in terms of the large sample standard errors of the two log-potencies concerned. As was observed in Part II of Cruickshank *et al.* (1953), these fiducial limits and significance tests take into account only the assay variation. Thus a significant difference between the contents of a particular tissue on the first and second days may reflect merely the biological variation between the two groups of rats used for the two experiments, and it would not necessarily follow that a single group of rats would show a difference in the same direction.

DISCUSSION

We have previously shown (Cruickshank *et al.* 1953) that the amount present in the liver of rachitic rats dosed with ergocalciferol represents about 60% of the total vitamin in the body on the first day after dosing and about 40% on the second day. This indicates that the liver is one of the main sites of the absorbed vitamin, at any rate during the short period investigated. This finding is at variance with that of Morgan & Shimotori (1943), who concluded that no one organ was specifically concerned with storage of vitamin D. Despite the low concentration in muscle and skin, these tissues contribute on the first day about 20%, and on the second day about 40% of the vitamin content of the entire body. Whether this high content in fact constitutes a store as suggested, at least for skin, by Vollmer (1939) is at present uncertain. The relatively high amount of vitamin contained in the kidneys is of some interest. In contrast to the liver content, which falls by about 1000 i.u. from the first to the second day, the content of the kidney does not decrease significantly. In consequence the concentration on the second day is somewhat greater than that of the liver. Since no vitamin D appears in urine (Cruickshank *et al.* 1953) it is likely that the kidneys act as a barrier and hence accumulate the vitamin. It is at present a matter of speculation whether this accumulation is of any metabolic significance. It may be associated with the mineral metabolism of the kidneys, or more specifically with the re-absorption of phosphorus by the kidney tubules (Harrison & Harrison, 1941). It may be noted that the concentration of the vitamin in the kidneys is considerably greater than the maximal concentration found in the intestinal tissue (Table 2) which also is concerned with the absorption of minerals. It has long been known that this process is influenced by vitamin D (Harris, 1932), but its exact mode of action is still unknown (Nicolaysen, Eeg-Larsen & Malm, 1953). If vitamin D acts *in situ* its presence in high amounts in the kidneys may be of significance.

Lungs and spleen had a relatively high concentration, as also had adrenals, in which, because of their active sterol metabolism, it may be of particular interest. Brain contained only small amounts, a finding not in accord with that of Houet (1946) who, however, used a colorimetric method of estimation.

The results given in Table 3 provide no evidence that bones and other tissues not examined in the present experiments contained any appreciable amount of the vitamin. Had they done so, the value obtained by summation of the vitamin content of the individual tissues examined in the present experiments would have been less than that obtained for the 'carcass'. In fact, however, the total content of the tissues, other than liver and intestines, obtained on the second day (890 i.u./rat), was significantly higher than the earlier estimate, 630 i.u./rat, for the whole carcass minus liver and intestines. This difference must be ascribed to biological variation between the two groups of animals used in these two series of experiments.

SUMMARY

1. The distribution of vitamin D has been studied in certain tissues of the rat on the first and second days after the oral administration of 1 mg. ergocalciferol (40 000 i.u.).

2. Muscle and skin together contained about 20% of the body vitamin on the first day and about

40% on the second day but the concentration was low, the estimate lying between 22 and 34 i.u./g.

3. The total vitamin D in the kidneys on the first and second days was estimated to be about 220 and 180 i.u., respectively. The corresponding concentrations were 270 and 200 i.u./g., which were of the same order as that found in the liver in previous experiments. It is suggested that the kidneys accumulate the vitamin by acting as a barrier to its elimination in the urine.

4. In lungs, spleen and adrenals, which contributed little to the total vitamin D content of the body, the concentration was of the order of 100 i.u./g.

5. From the results of the present experiment and those already published, there is no evidence that organs not examined (bones, etc.) contain any appreciable amounts of the vitamin.

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Quantitative Estimation of Glucose by Paper Partition Chromatography

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In the course of recent work it became desirable to develop a simple method for the quantitative estimation of reducing carbohydrates on paper chromatograms, which would be more accurate than that of direct photometry (Gustafson, Sundman & Lindh, 1951) or spot area elution (McFarren, Brand & Rutowsky, 1951), but less time-consuming than elution followed by micro-estimation (Flood, Hirst & Jones, 1948).

It was found that the coloured product formed by heating aniline hydrogen phthalate with glucose on paper could be quantitatively eluted and its concentration determined colorimetrically. Blass, Macheboef & Numez (1950) attempted elution with various organic solvents, but found it incomplete. The technique described below was however found to give reproducible results.

EXPERIMENTAL

Anhydrous glucose was dried to constant weight at 60° *in vacuo*. Quantities equivalent to 50, 100, 150 and 200 μg. were applied to Whatman no. 4 paper using an 'Agla' micrometer syringe. The spots were air-dried and the chromatograms developed 18 hr. by the ascending technique in the following solvent system: *n*-propanol–ethyl acetate–water (7:1:2, v/v) (Baar & Bull, 1953). The chromatograms were then air-dried and drawn through a solution consisting of 85% (v/v) aqueous *iso*propanol (400 ml.), phthalic acid (6·64 g.) and aniline (3 ml.). Hopkin & Williams 'AnalaR' aniline was redistilled before use. The reagent was kept in a dark bottle in the refrigerator. Excess of the solution was removed from the papers by means of a rubber roller. The air-dried sheets were placed on the bottom shelf of an electric oven and heated for 15 min. at 115±1°. The stained areas were cut out as rectangles allowing a suitable margin and choosing a size suitable to all concentrations. One unstained area of similar dimensions was used to provide a solvent blank. Each of these areas was extracted with glacial acetic acid (4 ml.) at room temperature for 24 hr. The typical absorption curve was established on a Unicam Spectrophotometer (SP. 600) and is shown in Fig. 1. By choosing a wavelength of 480 mμ., a calibration graph was established. This

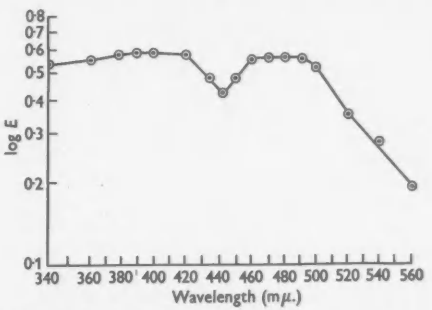

Fig. 1. Typical absorption curve of coloured product of reaction between glucose and aniline hydrogen phthalate. Colour developed on paper chromatogram and extracted with glacial acetic acid.

Table 1. *Factors influencing the colour yield*

Application area*	Reading	Time of heating (min.)	Reading	Extraction time† (hr.)	Reading	Stained area kept in dark (days)	Reading	Stained area kept in light (days)	Reading
1	0·238	5	0·223	0·25	0·081	1	0·239	1	0·240
2	0·242	10	0·240	0·5	0·100	2	0·241	2	0·236
3	0·240	15	0·239	2	0·176	3	0·238	3	0·221
		20	0·241	4	0·213	4	0·240	4	0·214
		25	0·239	6	0·220				
		30	0·235	8	0·230				
				12	0·241				
				24	0·240				
				2 days	0·238				
				5 days	0·221				
				7 days	0·214				

* Varied by increasing dilutions and represented as simple integers of the original volume.
† These results also indicate the stability of the extracted colour.

Table 2. *Recovery of glucose from chromatograms*

Glucose (μg.)	Recovery* (%)
25	102·0
50	99·8
100	100·0
150	97·0
200	98·5

* By comparison with glucose eluted and determined by the method of Jones & Pridham (1953). For details see Experimental section.

obeyed Beer-Lambert's law over a range of 0–300 μg. glucose with an optical density of 0·225 per 100 μg.

Various possible sources of error were investigated. These were influence of application area, time of heating, extraction time, ageing of colour reagent, different grades of paper and the stability of the colour complex in the presence or absence of light. Cells of 1 cm. depth were employed throughout.

To obtain a measure of the absolute accuracy of the partition chromatography employed, the above-described method was compared with an elution method, followed by micro-estimation. Two sheets were run in parallel, one stained and the corresponding areas cut from the other. The glucose was eluted by refluxing in a 'Quickfit' test tube containing 1 ml. of distilled water. The rolled paper was attached by means of a glass hook to the spiral inner coil tube of the condenser. The cold eluate was adjusted to 1 ml. and its glucose content estimated colorimetrically using benzidine (Jones & Pridham, 1953) at 290 mμ.

RESULTS

For a range of 50–200 μg. the coefficient of variation for single observations was found to be 0·72 %. This average was obtained from individual values of 0·57 % for 50 μg., 0·99 % for 100 μg., 0·71 % for 150 μg., and 0·68 % for 200 μg. The detailed results of the investigation of possible sources of error are shown in Table 1. Table 2 indicates the recoveries obtained by the new method in comparison with elution of glucose followed by micro-estimation.

Comparison of the mean values obtained using 'AnalaR' grade aniline manufactured by Hopkin & Williams and by British Drug Houses showed the following differences from the first mentioned: 50 μg., +0·55 %; 100 μg., +0·02 %; 200 μg., +0·14 %.

The effect of ageing of the colour reagent was investigated for a period of 2 weeks and the following results were obtained expressed as coefficients of variation for single observations. For 50 μg. the coefficient was 1·0 %, for 100 μg., 1·135 %, and for 200 μg., 0·35 %. Three runs were performed on Whatman no. 2 paper and compared with mean values obtained on no. 4 paper using Hopkin & Williams's aniline. No. 2 paper showed the following differences: for 50 μg., +0·75 %; for 100 μg., +0·55 %; for 200 μg., +0·44 %.

DISCUSSION

Although the above-described method is a general one, lacking specificity, it is well suited to carbohydrate estimation on chromatograms, since the lack of specificity is replaced by chromatographic separation. The high sensitivity of the method makes it possible to estimate very small quantities of glucose on a chromatogram employing a simple and reproducible technique.

SUMMARY

1. A simple colorimetric technique for the quantiative estimation of glucose eluted from chromatograms has been developed.

2. The results of investigating various sources of error are shown.

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